# THE EFFECT OF CYTOTOXIC DRUGS ON THE DNA METABOLISM AND PROLIFERATION OF LYMPHOBLASTS

BY

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To Mum and Dad,

who must have sometimes wondered what it was all about.

I only wish I knew enough to tell them,

thank you.

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## ABBREVIATIONS

| | |
|---|---|
| araA | adenosine arabinoside |
| araATP | adenosine arabinoside triphosphate |
| araC | cytosine arabinoside |
| araCDP | cytosine arabinoside diphosphate |
| araCMP | cytosine arabinoside monophosphate |
| araU | uracil arabinoside |
| araUTP | uracil arabinoside triphosphate |
| AMP | adenosine monophosphate |
| CTP | cytidine triphosphate |
| DEAE | diethylaminoethyl |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate where N can be cytosine, guanosine, adenosine or thymidine |
| DTH | delayed-type hypersensitivity reaction |
| DTT | dithiothreitol |
| dUMP | deoxyuridine monophosphate |
| EBV | Epstein-Barr virus |
| EDTA | ethylenediaminetetra-acetic acid |
| GVH | graft-verus host reaction |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| MIF | migration inhibitory factor |
| NTP | nucleoside triphosphate where N can be adenosine cytosine, guanosine or uracil |
| PBS | phosphate buffered saline (0.05 M phosphate buffer pH 7.2 containing 0.9% NaCl) |
| PEI | polyethyleneimine |

| | |
|---|---|
| PHA | phytohaemagglutinin |
| POPOP | 1,4-bis-2-(5-phenyloxazolyl)benzene |
| POP | 2,5-diphenyloxazole |
| PRPP | phosphoribosylpyrophosphate |
| RNA | ribonucleic acid |
| rNTP | ribonucleoside triphosphate (see NTP) |
| S.E. | standard error of the mean |
| TCA | trichloroacetic acid |
| THU | tetrahydrouridine |
| TRIS | Tris-(hydroxymethyl)aminoethane |
| XP | xeroderma pigmentosa |

## Summary

(A) The EBV transformed lymphoblastoid, Namalwa, cell line was used to investigate the effects of three cytotoxic agents on eukaryotic DNA replication. Cytosine arabinoside, an analogue of deoxycytidine, at a concentration of $5 \times 10^{-9}$ M was shown to inhibit DNA synthesis by 50% in the Namalwa cell line as measured by the incorporation of $^{3}$H-thymidine into acid precipitable material. A comparative study of the metabolism of cytosine arabinoside in transformed B and T lymphoblastoid cell lines and the protozoan, *T. pyriformis* was also undertaken. AraC has to be phosphorylated to its active form, araCTP, before it can exert a cytotoxic effect. Alternatively it can be inactivated by deamination to araU. The metabolites of araC were identified in each test system by using thin layer chromatography. The levels of araC kinase and araC deaminase were assayed. Both types of lymphoblastoid cells possessed high levels of araC kinase and no araC deaminase. *T. pyriformis*, on the other hand, had a low level of araC kinase and a high level of araC deaminase. The latter enzyme was inhibited 100% by $10^{-4}$ M tetrahydrouridine.

The effect of cytosine arabinoside on DNA replication was investigated using sedimentation analysis of DNA on alkaline sucrose gradients. The results indicate that araC inhibits DNA chain elongation. A comparative study of the effect of aphidicolin on DNA synthesis showed that $10^{-7}$ M aphidicolin inhibited DNA synthesis in Namalwa cells by 50%. The inhibitory effect of aphidicolin on DNA chain elongation was demonstrated using DNA sedimentation analysis on alkaline sucrose gradients.

The differing effects of cyclophosphamide, phosphoramide mustard and hydroperoxycyclophosphamide on DNA synthesis were demonstrated.

DNA synthesis in Namalwa cells was inhibited 20% by $5 \times 10^{-3}$ M cyclophosphamide, 65% by $5 \times 10^{-3}$ M phosphoramide mustard and 90% by $5 \times 10^{-3}$ M hydroperoxycyclophosphamide. Analysis of DNA on alkaline sucrose gradients indicated that the alkylating agents acted by cross-linking newly synthesised DNA giving unusual DNA intermediates but this hypothesis needs to be confirmed by further experiments.

(B) The immunosuppressive effects of the glucocorticoids, clobetasol propionate, clobetasone butyrate and 6-α-methyl prednisolone, on the delayed-type hypersensitivity response to the contact sensitising agent oxazolone was demonstrated in CBA mice. The inhibitory effects of cytosine arabinoside, aphidicolin and 6-α-methyl prednisolone on DNA synthesis in PHA stimulated human peripheral lymphocytes were demonstrated, 60% inhibition was obtained with $10^{-6}$ M 6-α-methyl prednisolone, $10^{-7}$ M aphidicolin and $10^{-8}$ M cytosine arabinoside.

Rat peripheral lymphocytes were stimulated to undergo blastogenesis with both PHA and an oxazolone-rat serum albumin conjugate. The DNA synthetic activity induced in both cases was inhibited by 6-α-methyl prednisolone. 34% inhibition was obtained with $10^{-4}$ M 6-α-methyl prednisolone in both the PHA and conjugate stimulated rat lymphocytes.

## Aims of the Project

There is a wide diversity of cytotoxic compounds that are potential or clinically used anti-cancer and immunosuppressive drugs each with differing mechanisms of action. The majority of these drugs exert their cytotoxic effect on all proliferating cells therefore a potential anti-cancer agent will have an effect on normal tissue as well as the malignant target tissue. Because of this many such drugs have an inhibitory effect on the cells of the immune system. In many cases those drugs which are used to treat diseases involving the immune system are also potent anti-cancer drugs. Many such drugs act by inhibiting DNA replication in proliferating cells. During the course of the work described in this thesis three types of cytotoxic agents were examined and their different mechanisms of action compared with respect to their effects on DNA replication in lymphoblasts.

The effects of two inhibitors of DNA synthesis, cytosine arabinoside and aphidicolin, were investigated with the aim of discovering at what point in the DNA replicative process they exerted their primary effect. Both are known inhibitors of DNA polymerase α and would therefore be expected to inhibit DNA chain elongation. However it has been proposed that araC may act primarily as an inhibitor of initiation so it was decided to investigate this possibility. The metabolism of araC in lymphoblasts and a protozoan model system was also studied with the aim that a study of araC metabolism could lead to a biochemical rationale for combination chemotherapy of araC with other drugs that could potentiate its effect as an inhibitor of DNA synthesis.

A comparative study was conducted on the inhibitory effect of the alkylating agent cyclophosphamide and its derivatives on DNA

replication. The two main aims of this study were (a) to investigate the differential effects of cyclophosphamide, phosphoramide mustard and hydroperoxycyclophosamide on DNA replication and to (b) investigate the hypothesis that these drugs act by cross-linking double stranded DNA thus preventing DNA replication.

The glucocorticoid steroids and their synthetic derivatives are used clinically as immunosuppressants and anti-inflammatory agents. The immunosuppressive effects of three steroids were investigated using an *in vivo* immunological assay with the aim of correlating activity to chemical structure. The assay used was a delayed-type hypersensitivity response to the contact sensitising agent oxazolone. An *in vitro* assay system using mitogen stimulated human and rat peripheral lymphocytes was set up in order to develop a comparable *in vitro* method for screening potential immunosuppressants. The aim was to develop this system further by stimulating the lymphocytes to undergo blastogenesis using an oxazolone-protein conjugate thus providing an *in vitro* analogue of the *in vivo* DTH assay.

# CHAPTER 1

## LYMPHOCYTE STRUCTURE AND FUNCTION

### 1.1 INTRODUCTION TO IMMUNOLOGY

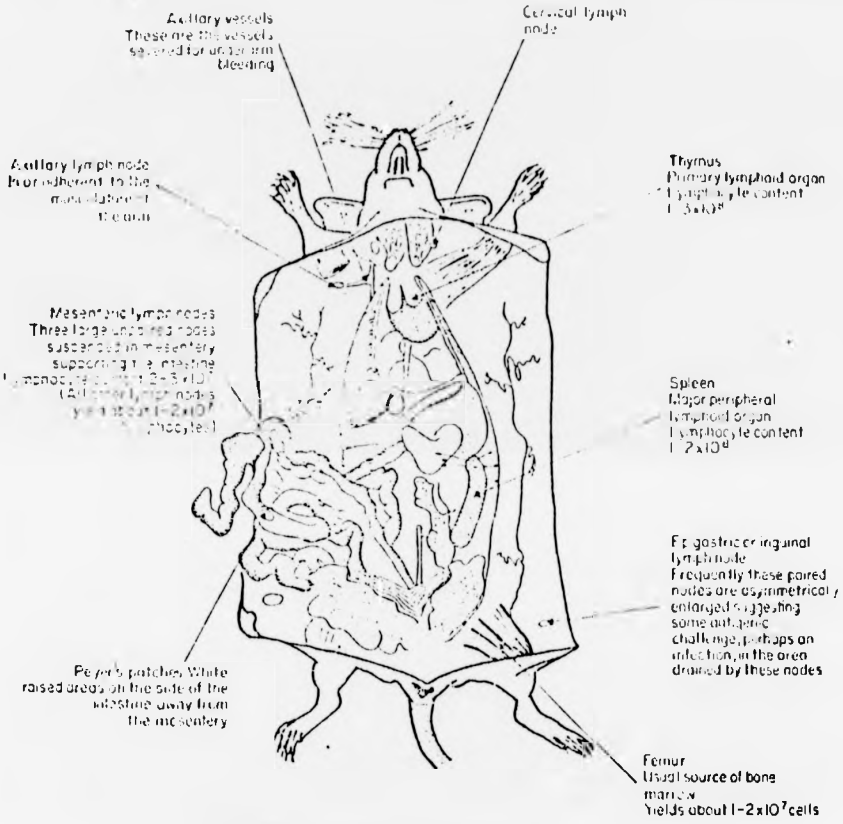
The vertebrate animal has a complex defence mechanism which affords it protection against invasion by foreign material. This protection is conferred upon the animal by the somatic tissues known as the lymphoreticular system (1). The fixed tissues of the lymphoreticular system consist of the bone marrow, thymus, spleen, lymph nodes, Bursa of Fabricius (only found in birds) and the gut-associated lymphoid tissue. The latter includes the tonsils, small intestinal Peyer's patches, the appendix and numerous areas of lymphocyte and macrophage accumulation within the lamina propria of the small intestinal villi and between the epithelial cells of the intestinal mucosal surface. The bone marrow is the site of origin of the pluripotent stem cells which ultimately differentiate into the various mobile cells of the lymphoreticular system. The thymus and Bursa of Fabricius are classified as primary lymphoid organs, the remaining tissues are classified as secondary lymphoid tissues.

The mobile cells can be sub-divided into myeloid and lymphoid cells. The myeloid cells consist of the granulocytes, monocytes and macrophages and these all have phagocytic properties. Phylogentically these are the most primitive cells in the animal's defence system and are the main defence mechanism in invertebrates. The lymphoid cells consist of the B and T lymphocytes and their sub-sets and immunology is generally regarded as being the study of the structure and function of these cells. It is these cells that confer the property of specific immunity upon an animal.

The B lymphocytes are formed in the Bursa of Fabricius in birds and in the "Bursa equivalent" in mammals. The "Bursa equivalent" has not been fully defined in mammals but is probably the bone marrow. The host's B cells respond to invasion by a foreign material (antigen), e.g. foreign protein or bacterium, by producing a specific protein (antibody). Antibodies are a species of protein known as immunoglobulins and have two major functions, one is to bind the antigen and the other to elicit secondary biological phenomena resulting in destruction of the antigen. This response is known as the humoral immune response.

T lymphocytes are formed in the thymus and are responsible for what is known as cell-mediated immunity. They can be divided into a number of subsets with different properties. They do not produce antibodies like the B lymphocytes but interact with the other cells of the lymphoreticular system in a very subtle fashion thus controlling the immune response. They can promote or suppress B cell activity, they secrete a variety of lymphokines and are also capable of destroying invading cells by the secretion of toxins. They are involved in the delayed-type hypersensitivity response (DTH) and in the graft-versus host reaction (GVH).

One other defence mechanism that animals possess is a group of heterogenous, low molecular weight proteins known as the interferons which are produced by infected host cells to protect non-infected cells from viral infection (2). It was first thought that interferon was a single protein but it has now been shown that there are several types of interferon. Lymphocytes are known to produce at least two types of interferon and possibly a third. Fibroblasts have also been shown to be capable of producing interferon. The latter can be distinguished




**Lymphocyte sub-populations of lymphoid organs**

| Organ | % T lymphocytes | % B lymphocytes | % 'Null' cells |
|---|---|---|---|
| Thymus | 97 | 1 | 2 |
| Lymph node | 77 | 18 | 5 |
| Spleen | 35 | 38 | 27 |
| Blood | 24 | 70 | 6 |
| Thoracic duct lymph | 80 | 19 | 1 |

**Definition of cells in figure**

*T lymphocyte.* Thymus-derived small lymphocyte, detected by presence of *θ* antigen and absence of Ig on surface membrane.

*B lymphocyte.* Bursa-equivalent-derived small lymphocyte, detected by presence of Ig and absence of *θ* antigen on surface membrane.

*'Null' cell.* Small mononuclear cell, not *θ* or Ig on surface membrane may or may not be a cell of the lymphoid series, and probably of more than one cell lineage.

Fig 1.1 Mouse lymphoid organs and their lymphocyte populations

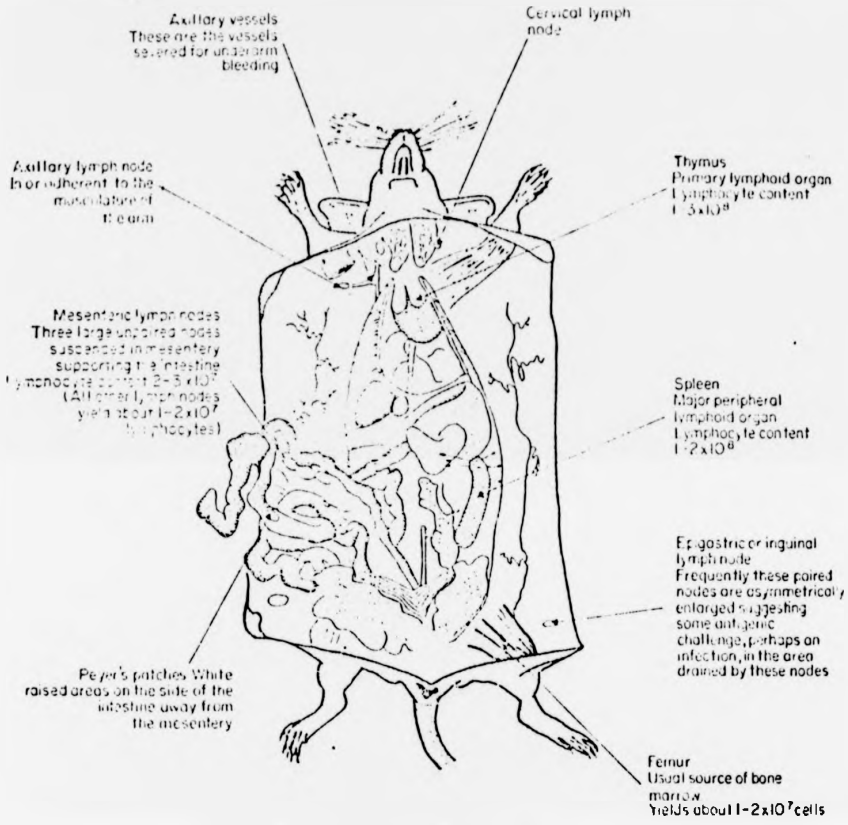



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Fig 1.1 Mouse lymphoid organs and their lymphocyte populations

from lymphocyte interferons as it is a glycoprotein. It seems as if interferon acts in response to the presence of double-stranded RNA, the replicative form of RNA produced by viruses. Work with the artificial ds.RNA poly I.poly C. has shown that interferon inhibits protein synthesis in two ways. One method is the phosphorylation of the protein synthesis initiation factor eIF.2 by an interferon mediated protein kinase. The second involves degradation of messenger RNA by an endonuclease which is activated by the unusual nucleotide $pppA^{2'}p^{5'}A^{2'}p^{5'}A$, abbreviated to 2.5A (3). Recent work on interferon has suggested that it might be a possible cancer chemotherapeutic agent as well as an anti-viral agent.

## 1.2 B AND T CELL DEVELOPMENT AND DIFFERENTIATION

All blood cells, erythrocytes, myeloid cells and lymphoid cells, are derived from primitive, pluripotent haematopoietic stem cells formed in the bone marrow. During embryogenesis stem cells are produced in the yolk sac. The site of stem cell production then changes to the foetal liver and finally to the bone marrow where it remains throughout adult life. These stem cells differentiate to form lymphocytes in the primary lymphoid organs. The differentiated cells then migrate to the secondary lymphoid organs from where they enter the recirculating lymphocyte pool in the blood and lymphatic vessels.

Haematopoiesis, the development and differentiation of the blood-forming cells, is a complex process involving subtle interactions between the different cell types and haematopoietic tissues (4). All the different cell types originate from the same pluripotent stem cell. Evidence for a single stem cell has come from experiments in mice in which chromosome markers were induced in donor bone marrow cells by irradiation. Myeloid spleen colonies and B and T lymphocytes in reconstituted animals had the chromosome marker of a common precursor.

Evidence for the existence of pluripotent stem cells in man has come from the appearance of the Philadelphia chromosome in cells of the granulocytic, mononuclear phagocytic and erythroid lines in patients with chronic myeloid leukaemias.

The process of commitment of the stem cells to a particular line of development is poorly understood. It is known that this process does come under hormonal control from polypeptide hormones known as haematopoietins (5). However a stem cell is not in itself committed to a line of development simply by exposure to one of the specific haematopoietins. For example the triggering signal for erythrocyte production is the balance between tissue-oxygen needs and the capacity of the oxygen delivering system. A stem cell is not responsive to the erythrocyte producing haematopoietin, erythropoietin, until at some point in its development it forms an erythropoietin responsive stem cell which is then committed to development along the erythroid pathway, commitment may therefore be defined as the acquisition of responsiveness to a specific haematopoietin. Haematopoietins for other cell lines are the CSA factor (colony stimulating activity) for granulocytes, thymopoietin for T lymphocytes and bursopoietin for B lymphocytes, produced in the thymus and Bursa respectively. A non-selective inducing agent found in all tissues is the polypeptide ubiquitin, this is not a physiological inducing hormone. All these polypeptides act by increasing cyclic AMP levels in the precursor cells. Other agents have also been shown to be capable of non-selectively inducing lymphocyte differentiation. These include cyclic AMP, dibutyryl cyclic AMP, adrenaline, bacterial endotoxin, cholera toxin and poly(A:U) all of which act by raising intracellular cyclic AMP levels. All of these agents act as positive controls in haematopoietic development.

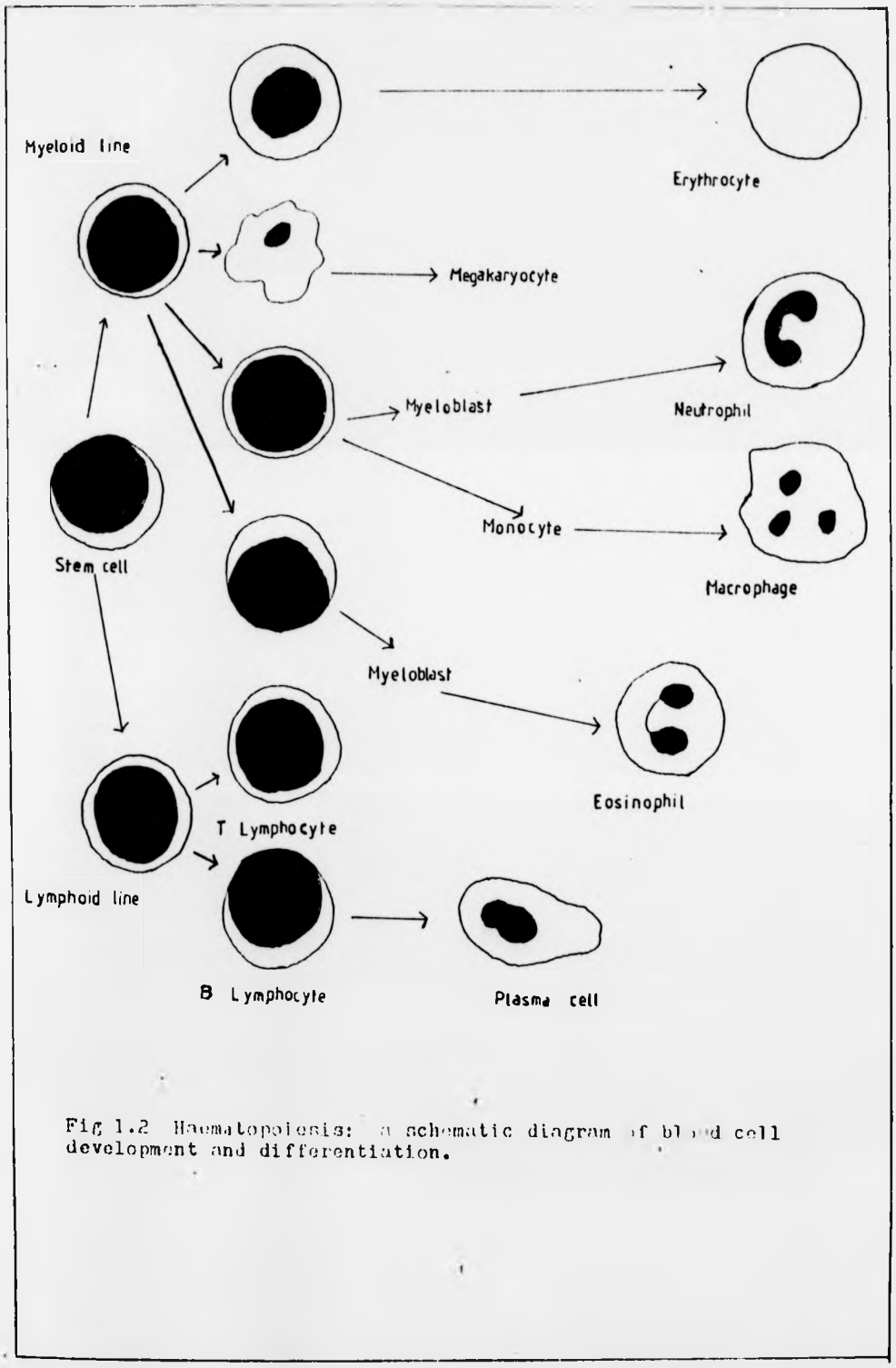



Fig 1.2 Haematopoiesis: a schematic diagram of blood cell development and differentiation.

As well as the positive controls there are a number of negative feedback controls and interactions between the different cell populations. Negative feedback control is thought to be produced by chalones. Chalones are defined as tissue specific, species non-specific, products of differentiated cells that selectively inhibit early cells of the same lineage. This type of negative feedback control has been postulated for granulocytopoiesis though its existence has not been fully established. Another form of negative control of myeloid cell development .that has been reported is the existence of neutrophil derived factors that inhibit cells producing CSA. Prostaglandin involvement has also been demonstrated, $PGE_1$ and $PGE_2$ have been shown to be inhibitors of granulocyte proliferation whereas F prostaglandins are stimulatory.

There is also extensive interaction between the different cell lines, e.g. CSA is produced by lymphocytes, monocytes and macrophages, and prostaglandins are produced by monocytes and macrophages. It has also been suggested that erythropoiesis is modulated by T cell interaction. Another form of cell-cell interaction is that between proliferating haematopoietic cells and the stromal cells of the blood forming organs. The rate of stem cell replication and differentiation is partly determined by factors derived from the organ in which the cells are situated. These factors are collectively referred to as the haematopoietic inductive microenvironment and may be under some form of genetic control.

## 1.3 B CELL STRUCTURE AND FUNCTION

The differentiation of B lymphocytes from pluripotential stem cells to fully mature antibody-secreting cells can be divided into two stages. The first stage consists of the differentiation of the cells within the primary lymphoid organs (see Section 1.2). This stage is known as

clonal development and results in the formation of immunocompetent B lymphocytes. These cells carry specific cell surface markers that can be used to differentiate them from T lymphocytes. These markers are very useful as to all intents and purposes all lymphocytes look the same when viewed under the light microscope having a characteristic round appearance consisting of very little cytoplasm surrounding a large nucleus which occupies most of the cell volume. The cell surface markers fall into three categories and can be used to identify sub-populations of B lymphocytes in the circulation. The principle categories are surface immunoglobulin molecules, receptors for the Fc portion of the immunoglobulin molecule and cells with receptors for the third component of complement ($C_3$). Often B lymphocytes express two of these markers and occasionally three. These markers may represent different stages in the sequential maturation of B lymphocytes or they may denote discrete populations of B lymphocytes (6).

The second stage of B lymphocyte development is the antigen-induced differentiation of B lymphocytes to mature plasma cells and memory cells. The second stage is referred to as clonal selection by antigen. When the immunoglobulin bearing B cell binds to an antigen the cells are stimulated to undergo either clonal expansion giving rise to daughter memory cells for that particular antigen or they differentiate directly into mature, specific antibody-secreting plasma cells. The memory cells have a long life span of several months or years in contrast to the plasma cells which are relatively short lived having half-lives that can be measured in days.

An antigen can be described as an invading foreign particle within the host animal which elicits an immune response. The invading particle has to fulfil certain requirements for it to have antigenic properties. It must be a large particle with a molecular weight generally greater

than 5,000. The potential antigen must have a rigid structure and the antigenic determinant (that part of the molecule recognised by the antibody) has to be of a consistent shape and charge pattern. Antigens must also have a high degree of internal complexity and are often multivalent, i.e. having more than one antigenic determinant. Small molecules can be coupled to large carrier molecules such as proteins to produce antigens. Small molecules that can act in this fashion are called haptens and are non-immunogenic when not coupled to a carrier.

Antibodies are a class of proteins known as immunoglobulins. Immunoglobulins are made up of equal numbers of heavy and light polypeptide chains with a general formula $(H_2L_2)_n$. These chains are held together by non-covalent forces and interchain disulphide bridges. Each chain is made up of a number of loops called domains. The 'N' terminal domain has a variable amino acid sequence compared with the other domains. This variable region represents the antigen binding site and confers specificity upon the antibody. As well as a complex two-dimensional structure immunoglobulins have a complex three-dimensional structure. The polypeptide chains of each domain are folded into a series of anti-parallel folds forming two surfaces held together by a disulphide bridge. This basic shape allows non-covalent interactions between pairs of domains thus holding the structure rigid.

As well as binding to specific antigens the immunoglobulins have other important biological, or adjunctive, functions to perform as part of their role in the humoral immune response. One of these functions is the fixation and activation of the complement system. The complement system is a group of serum proteins that acts as the primary humoral mediator of the antibody-antigen reaction. The activation of

the complement system procedes via a cascade of triggered enzyme systems that ultimately cause cell lysis. Another important biological function is the transfer of immunoglobulins across epithelial membranes. This is of importance in providing antibodies for the external surfaces of the body e.g. the alimentary, respiratory and reproductive tracts. They can also be transferred across the placenta from mother to foetus, a function which is of particular importance in the protection of the newly-born.

## 1.4 T CELL STRUCTURE AND FUNCTION

T lymphocytes are the primary mediators in cell mediated immunity and can be distinguished from B lymphocytes by differences in their surface membrane. One obvious difference is the lack of immunoglobulins on the surface membrane of T cells. There are also a number of distinctive cell surface markers including certain alloantigens. These have been mainly studied in mice because of the availability of a large number of inbred strains. Such antigens are the Thy 1 and Ly 1,2,3 and 5 antigens, the other member of the series, Ly 4, is also present on B lymphocytes. These antigens can be identified by their reaction with specific antisera. Human T lymphocytes also possess surface receptors for sheep red blood cells. The sheep erythrocytes bind to the T cell surface forming a rosette of red cells around a central lymphoid cell. The rosette test has proved to be a useful marker for human T lymphocytes.

The classification and characterisation of the many T cell sub-populations is very complex because their morphological differences are somewhat unclear. They are best distinguished by their specific functions. The sub-populations of T lymphocytes are helper cells, suppressor cells, cytotoxic T cells, killer cells and natural killer cells (6). The helper T lymphocytes function as "helpers" in inducing

antibody responses. The synthesis of antibodies by B lymphocytes is critically dependent on the presence of adequate numbers of helper cells. The opposite function is performed by suppressor cells which act by limiting specific immune responses such as the antibody-antigen response.

Cytotoxic T cells are specifically sensitised to kill target cells bearing antigens that have been induced by virus, bacterial or parasitic infections. The cytotoxic response of T lymphocytes to persistent viral infection is of great importance. Most individuals are infected at some time in their lives with *Herpes simplex*, cytomegalovirus or Epstein-Barr virus. Normally transient illness occurs after primary infection but after that persistent infection is of little clinical consequence. Defects in this aspect of immunity can have serious consequences e.g. children with severe cytomegalovirus infections or lymphoproliferative disorders such as infectious mononucleosis and lymphoma after EBV infection.

Killer cells are a distinct sub-population of lymphoid cells capable of killing target cells sensitised with antibodies to their cell surface antigens. This phenomenon is called antibody-dependent cell-mediated cytotoxicity (ADCC). Killer cells have some B cell characteristics, such as receptors for $C_3$ and surface immunoglobulins, and some T cell characteristics such as rosette formation with sheep erythrocytes. Killer cells therefore do not fit easily into any category though they appear to be of thymic origin.

Natural killer cells (NK) have been identified as a population of lymphoid cells capable of lysing or damaging a variety of malignant target cells *in vitro*. This has lead to suggestions that they may play a role in tumour surveillance (7). However more recent evidence suggests that NK cells play an integral role in T cell and non-T cell

interactions and may have an immunoregulatory role (8). It has been shown that NK activity can be augmented by interferon and that their primary role may be as a surveillance mechanism directed against viruses. Like killer cells they appear to be of T cell lineage having weak affinity receptors for sheep erythrocytes though this is a controversial point as they also possess Fc receptors, a characteristic of B lymphocytes.

## 1.5 CELL INTERACTIONS IN THE IMMUNE RESPONSE

The immune response is under strict regulatory control via a series of cellular interactions. One such example is the control of haematopoiesis (see Section 1.2). The control of both humoral and cell-mediated immunity is also governed by the interactions of different lymphocyte populations. The humoral, B lymphocyte mediated, immune response is controlled by T lymphocyte helper and suppressor cells (see Section 1.4). The cell-mediated immune response is itself controlled by the release of chemical mediators known as lymphokines (6).

Lymphokines are non-immunoglobulin mediators produced by activated lymphocytes which produce their effect by acting on the cell-mediated immune response (9). There are many different lymphokines and it is not yet known exactly what activity each lymphokine possesses and much work has still to be done in the physiology and biochemistry of lymphokine secretion (10). It is known that they are released in minute amounts and that their activity is relatively intense.

One of the first lymphokines to be discovered was macrophage migration inhibition factor (MIF). Its function is to inhibit the migration of normal macrophages. A number of other lymphokines have been demonstrated in mouse sera, these include a bacteriostatic factor, a tumour inhibiting factor, a lymphotoxin, a mitogenic factor and a

mixed lymphocyte reaction suppressor factor. Both B and T cells seem to be involved in lymphokine release. In the case of MIF evidence suggests that T cells are initially important in the antigen sensitisation stage and once sensitised T and B cells interact to produce MIF. The interaction of interferon with lymphocytes also suggests its involvement in immunoregulation. All this emphasises the complexity of immunoregulatory control and the large degree of integration between the various components of the immune system.

## 1.6 THE USE OF IMMUNOSUPPRESSIVE DRUGS

Immunosuppressive drugs are agents which depress or suppress the development of one aspect of the immune response (11, 12). The majority of these drugs exert their effect by interfering with the growth of rapidly dividing cells and are therefore not specifically directed against immune function. Immunosuppressive agents are often active at doses close to their toxic level and therefore can produce a number of undesirable side effects. This means the use of such drugs has to be carefully considered and it is essential to determine the best administration regimen which will permit maximum immunosuppressive activity with the least toxicity.

The mechanisms of the different types of immunosuppressive agents differ widely. Alkylating agents such as chlorambucil and cyclophosphamide act by crosslinking macromolecules such as DNA thus preventing cell replication. The antimetabolites such as cytosine arabinoside and methotrexate resemble natural enzyme substrates and compete with the natural substrates for enzyme active sites. Cytosine arabinoside is an inhibitor of DNA polymerase $\alpha$ and methotrexate is an inhibitor of tetrahydrofolate reductase which results in the depletion of tetrahydrofolate, a cofactor for the enzyme thymidylate synthetase. The mechanism of action of corticosteroids is still unresolved but they

appear to act by suppressing DNA and RNA synthesis. Some antibiotics and plant alkaloids and cytotoxic antisera to lymphocytes also act as immunosuppressants.

Immunosuppressive drugs are used as chemotherapeutic agents in a number of diseases involving the immune response (12, 13). They are widely used in the treatment of inflammatory diseases though sometimes, as in the case of corticosteroids, the anti-inflammatory effect is not necessarily linked to the immunosuppressive effect. Cyclophosphamide and chlorambucil have been used in the treatment of systemic lupus eryethematosus. Rheumatoid arthritis has been treated with methotrexate, cyclophosphamide and azathioprine. Psoriasis has been effectively treated with methotrexate and alkylating agents.

Another major use of immunosuppressants is in transplant surgery. Here their ability to suppress the immune response is used to prolong graft survival in patients receiving organ transplants and skin grafts. The corticosteroids have been particularly useful for this purpose. A new compound which has enormous potential in this field is the recently discovered fungal metabolite cyclosporin A which is a specific inhibitor of cell-mediated immunity.

Many drugs with immunosuppressive activity are used in the treatment of tumours and cancers as a result of their non-specific action on rapidly metabolising cells (14, 15). In many cases of cancer chemotherapy one of the undesirable side effects of the drugs used is their immunosuppressive effect. It is this difficulty in finding selective drugs which makes cancer chemotherapy so difficult as any drug affecting cell metabolism will also act on normal cells as well as malignant cells. It is the seriousness of these diseases which justifies using such drastic treatment. One form of cancer in which the immunosuppressive property is desirable are those cancers of the immune system i.e. the

leukaemias and lymphomas discussed below.

## 1.7 LEUKAEMIAS AND LYMPHOMAS

Leukaemias and lymphomas are both malignant neoplastic diseases of the cells involved in the immune response and usually arise from the clonal proliferation of one cell type. Their main difference is anatomic rather than pathogenic e.g. chronic lymphocytic leukaemia and lymphocytic lymphoma may be abnormalities of the same B cell (16). Lymphomas are solid tumours and are anatomically restricted to the lymphoid tissues. The two major categories of lymphoma are the Hodgkin's and non-Hodgkin's lymphomas. There is no completely satisfactory definition for Hodgkin's lymphomas but they can be morphologically defined as malignant neoplasms which arise in some lymph nodes, often spreading to others, composed of neoplastic and normal cells intermingled in a uniquely complicated way. Non-Hodgkin's lymphomas are defined as all other solid lymphoreticular neoplasms and they consist almost entirely of neoplastic cells.

Leukaemias differ from lymphomas in that a significant number of the malignant cells are constantly present in the blood or bone marrow. Leukaemia is associated with abnormal leucocyte counts, anaemia and thrombocytopenia. Death usually occurs as a result of infection or haemorrhage. The two major classifications of leukaemia are the myeloid and lymphoid leukaemias derived from neoplastic myeloid and lymphoid cell lines respectively. These can be further sub-divided into the acute and chronic leukaemias. The acute leukaemias are rapidly fatal diseases, the average time interval from onset to death in untreated cases being two to four months. The chronic leukaemias are diseases of longer duration of around three to six years between onset and death. These classifications must be considered as generalisations as not all lymphomas and leukaemias can be so neatly

characterised (17).

Many leukaemias and lymphomas exhibit visible, non-random, chromosomal rearrangements (18, 19). About 90% of chronic myeloid leukaemias show a translocation from chromosome 22 to 9 which creates the shortened 22 chromosome known as the Philadelphia chromosome. There is frequent involvement of chromosome 14 abnormalities in B cell neoplasms as in Burkitt's lymphoma. In murine T cell leukaemias trisomy at chromosome 15 is often observed. It is thought that these translocations may be the specific genetic change required for the transition of pre-neoplastic cells to neoplastic cells. It is not yet certain what the initiators for leukaemia and lymphoma development are. In the case of Burkitt's lymphoma it is known to be a virus, the Epstein-Barr virus (EBV); this is also the case in some murine leukaemias (20). There is no direct evidence for virus involvement in human leukaemias though the possible involvement of RNA viruses has been indicated by the presence of the enzyme reverse transcriptase in some leukaemic cell types (21, 22, 23). Other possible initiators are chemical carcinogens and radiation.

There are a number of different approaches to therapy, the aim of which is to induce remission a state in which all clinical and laboratory signs of the disease have disappeared. Types of therapy include bone marrow transplants, radiation therapy, chemotherapy and immuno-therapy. The latter can take the form of immunisation against a tumour cell type, passive transfer of antisera or cells with anti-tumour activity and non-selective stimulation of the immune response with drugs (24). Chemotherapy often takes the form of multi-drug regimens consisting of a number of drugs acting in different ways (25, 26, 27). Chemotherapy is often supplemented with radiation therapy e.g. in the treatment of acute leukaemias irradiation of the central nervous system is carried out to prevent the onset of meningitis (28). Many

of the drugs used are the same as those described in Section 1.6. Cyclophosphamide is used to treat Burkitt's lymphoma and cytosine arabinoside is used in combination with drugs such as daunorubicin, prednisolone and methotrexate in the treatment of myeloid leukaemias.

# CHAPTER 2

# EUKARYOTIC DNA SYNTHESIS

## 2.1 HISTORICAL PERSPECTIVE

The complexity of the mechanism of DNA synthesis has become increasingly apparent since the double helical structure of DNA was first elucidated by the X-ray diffraction studies of Watson and Crick. The DNA molecule consists of two polynucleotide chains intertwined in a helical fashion. The backbone of the two chains consists of deoxyribose units linked by 3', 5'-phosphodiester bridges. The two chains are held together by hydrogen bonding between complementary base pairs of cytosine and guanine, and adenine and thymine. Each stand has a polarity caused by the phosphodiester bond between C-3' and C-5' atoms of two neighbouring nucleosides, the two complementary strands having opposite polarities.

A great deal of our understanding of the biosynthesis of this macromolecule has come from work on prokaryotes, mainly *E. coli* which has a single circular chromosome a much simpler system when compared to the multi-chromosome system of eukaryotes. It was by using this organism that Meselsohn and Stahl demonstrated the semi-conservative nature of DNA synthesis, i.e. that newly synthesised DNA molecules consist of one newly synthesised daughter strand and one parental strand. The opposite polarities of the two strands presented problems when explaining the mechanism of DNA replication, either the two strands are synthesised by two separate polymerizing activities or by some more complicated process. This problem was solved by Okazaki who found that one strand, the leading strand, is synthesised continuously in the same direction as the movement of the replication

fork whereas the other, lagging, strand is synthesised discontinuously in short pieces in the opposite direction. These short pieces are then joined together to give net synthesis in the same direction as the leading strand (29). The direction of synthesis has been shown to be in the 5' to 3' direction along the new DNA strand.

From this it becomes apparent that the enzymatic machinery involved in DNA synthesis is extremely complex (30). Besides the existence of a polymerizing activity there has to be a mechanism for unwinding and separating the strands of the helix, for priming and initiating the process, ligating the Okazaki fragments on the lagging strand and for termination. The whole mechanism has to proceed at a rate compatible with cell growth and close to 100% accuracy. The process in *E. coli* is known to require at least a dozen different proteins all involved with the control and synthesis of new DNA molecules.

The first of these proteins to be discovered was the DNA polymerase I of *E. coli*. This enzyme can use nicked duplex DNA, duplex DNA containing large gaps or a primed single strand of DNA as a template. As well as possessing polymerizing activity it also has 3' to 5' exonuclease activity and 5' to 3' exonuclease activity. It was originally thought to be the major enzyme of DNA replication until a mutant was isolated defective in this enzyme yet still capable of replication. This lead to the discovery of DNA polymerase III. This enzyme is made up of at least four subunits and exists in two main forms, the polIII enzyme and the holoenzyme consisting of polIII and copolymerase III. The holoenzyme is the functional enzyme in *E. coli* DNA synthesis. It can only use single stranded DNA as a template and possesses 3' to 5' exonuclease activity. This exonuclease activity is part of a proof-reading mechanism for removal of mismatched bases.

A third DNA polymerase has been found in *E. coli*, polymerase II. The role of this enzyme is not clear but it may be involved in DNA repair.

The other proteins involved in *E. coli* DNA synthesis have been identified and their roles established by using bacteriophages such as ØX174, coliphage M13 and G4 as probes. These phages have much smaller and simpler chromosomes than *E. coli*. These phages depend on the enzymes of *E. coli* for their replication and each phage has been used to investigate a different part of the DNA synthesis machinery of *E. coli*. The life cycle of ØX174 is shown in Fig. 2.1 and a list of the proteins involved in the conversion of ØX174 SS-DNA to RF-DNA is shown in Fig. 2.2. The cisA protein is peculiar to the life cycle of ØX174. The other proteins can all be assigned a role in the replication of *E. coli* DNA (see Fig. 2.3) (31).

A leading strand started at the origin of replication of the chromosome advances continuously, its progress being made possible by the unwinding action of the rep protein. Binding protein stablises the single stranded DNA so it can act as a template while the bared opposite strand becomes the template for the discontinuous synthesis of the lagging strand. Delivery of the promoter dnaB protein by the prepriming proteins dnaC, protein i and protein n prepares the template for priming. Proteins dnaB and dnaC are used stoichiometrically whereas proteins i and n are used catalytically. The primase, dnaG, synthesises a short segment of RNA to serve as a primer for the covalent extension by DNA polymerase III holoenzyme. Elongation may average 1000 nucleotides or so. Progressive movement of the dnaB protein in the 5' to 3' direction of the leading strand enables it to follow the progress of the replicating fork also enabling primase to make successive discontinuous initiations of the lagging strand. DNA polymerase I removes the RNA primer by its 5' to 3' exonuclease activity and fills in the gaps on the lagging strand. The ends of the discontinuous

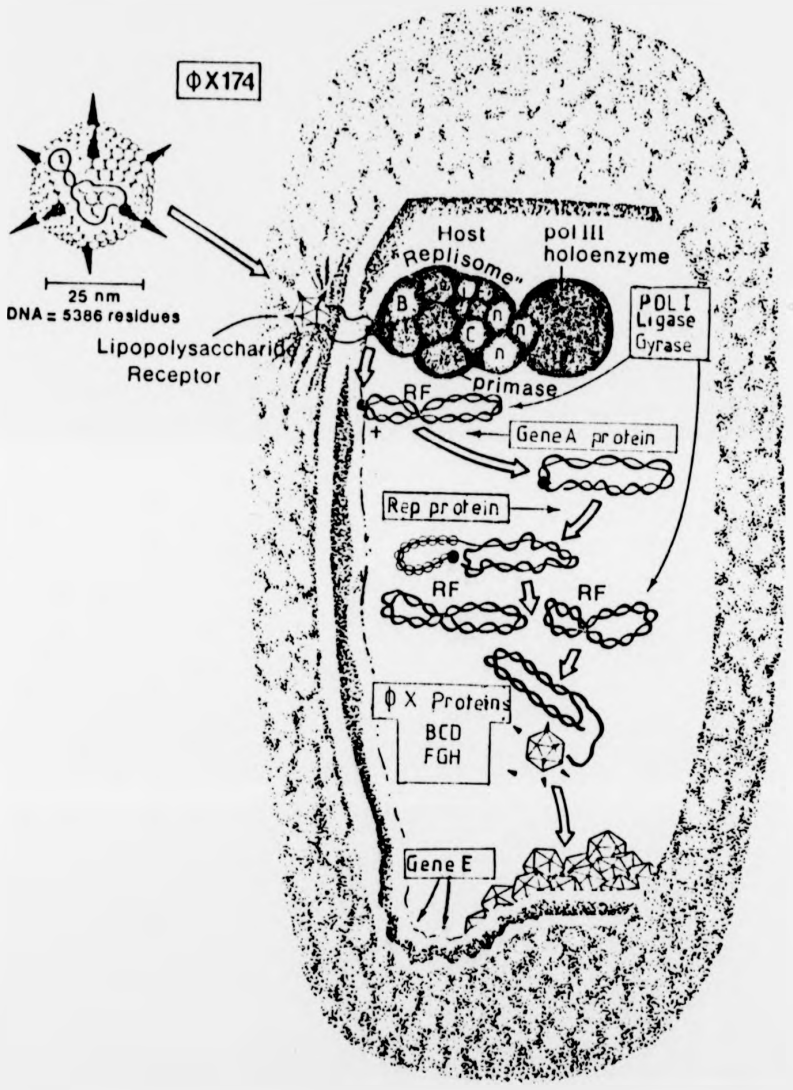



Fig 2.1 Life cycle of ØX174. The circular single stranded DNA chromosome is copied to give the double stranded, replicative (RF) form. The original strand is designated (+) and the newly synthesised strand (-). The (-) strand is used a template to synthesise more (+) strands and hence new phages.

(from "DNA Replication" by A Kornberg, W H Freeman and Company)

| Polypeptide | Mass kdal | Subunits | Function | Unamplified yield molecules/cell | Unamplified yield mg/kg[c] | Amplification |
|---|---|---|---|---|---|---|
| SSB | 74 | 4 | single strand binding | 300 | 20 | |
| protein i | 80 | 4 | prepriming | 150 | 0 5 | |
| protein n | 25 | 1 | prepriming | | | |
| protein n′ | 75 | 1 | site recognition, ATPase | 80 | 0 3 | |
| protein n″ | 11 | 1 | prepriming | | | |
| *dnaC* | 29 | 1 | prepriming | | | |
| *dnaB* | 250-300 | 4-6 | mobile promoter, ATPase | 20 | 0 3 | 10-100 |
| primase | 60 | 1 | primer formation | 100 | 0 2 | |
| holoenzyme′-α | 140 | 1 | | | | |
| β | 40 | 1 | | | | |
| γ[b] | 52 | 1 | | | | 10 |
| δ | 32 | 1 | synthesis | 20 | 0 5 | |
| ε | 25 | 1 | | | | |
| θ | 10 | 2 | | | | |
| pol I | 109 | 1 | | 300 | 10 | 70 |
| ligase | 74 | 1 | ligation | 300 | 10 | 500 |
| gyrase | 400 | 4 | | | | |
| *nalA*(A) | 210 | 2 | supertwisting | | | |
| *cou*(B) | 190 | 2 | | | | |
| *rep* | 65 | 1 | helicase | 50 | 0 6 | 10 |
| dUTPase | 64 | 4 | dUTPase | 350 | 3 | |

[a] Mg protein/kg wet weight of cells
[b] *dnaZ* (γ polypeptide)
[c] See Table 5 3 for more details
[d] Normal protein level was increased this many times by introducing a plasmid or phage vector containing the encoding gene

Fig 2.2 The proteins used in ØX174 DNA replication in E. coli host cells.

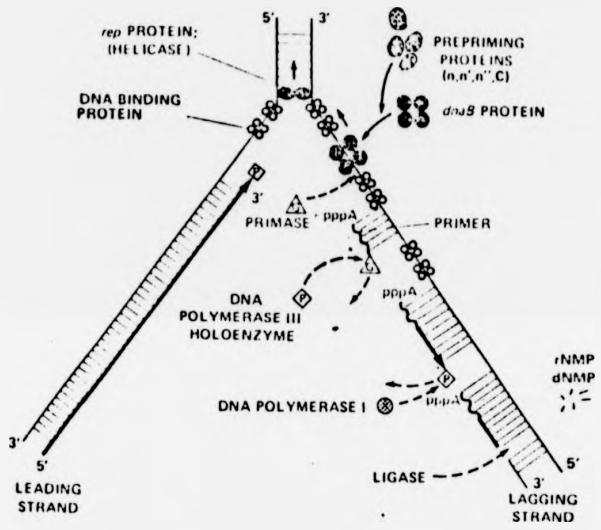



Fig 2.3 Hypothetical scheme for the replication fork of the E. coli chromosome.

segments are then sealed by DNA ligase activity uniting the nascent fragments into an intact duplex.

## 2.2 ORGANISATION OF THE EUKARYOTIC CHROMOSOME

The eukaryotic chromosome is much larger than a prokaryotic chromosome. This increase in size is concommitant with an increase in complexity and organisation. The prokaryotic chromosome replicates as a whole unit starting at a unique origin and proceeding until the whole unit has been copied, this unit of replication is called a replicon. Eukaryotic chromosomes do not replicate as single elements. Instead different sections of a chromosome are replicated at different periods of the S phase of the cell cycle, i.e. eukaryotic chromosomes contain more than one replicon or replication unit. This form of DNA replication requires a more sophisticated level of organisation than exists on a prokaryotic chromosome (32).

The DNA of the eukaryotic chromosome is organised into repeating structural nucleoprotein units called nucleosomes of about 200 base pairs in length complexed with histones (33). The nucleosome can be sub-divided into a core particle and a linker region. The nucleosome core consists of approximately 140 base pairs wound around a histone octamer containing two each of the histones H2A, H2B, H3 and H4. Histone H1 associates with a further 60 base pairs to form the linker and appears to play a role in the higher order packing of chromatin. There is also a complex range of local variations in the distribution of non-histone proteins along the length of the chromosome.

Nucleosomes can be formed *in vitro* from histones and DNA by dialysis against salt solutions the speed of formation depending on ionic concentration. Two proteins have been found in the eukaryotic nucleus which allow nucleosome core assembly to proceed rapidly *in vitro*,

these are topoisomerase I (nicking-closing enzyme) and nucleoplasmin, an acidic, thermostable protein (34). It is thought that these proteins may act together by competing against or masking the strong electrostatic interactions between histones and DNA thus allowing assembly to occur without aggregation and precipitation as occurs in salt solutions. There is as yet no direct evidence that these two proteins act as assembly factors *in vivo* but indirect evidence seems to support their involvement in nucleosome assembly. The nucleosome structure is integrated into the helical structure of the DNA molecule. It is thought that the helix is then super-coiled to form the close packed structure of the eukaryotic chromosome.

The control of chromosome replication is organised on several levels all of which may be regulated in part by chromatin structure (35). There is a degree of temporal control at the level of the chromosome in that certain sections of the chromosome are replicated at defined times in S phase. This degree of control is more apparent in lower eukaryotes. The next level of control is exerted on a cluster of 100 replication units or less. Such clusters replicate synchronously as a whole section of DNA within the chromosome. The third level of organisation is the replication unit itself. A replication unit is a stretch of DNA replicated by two adjacent growing points which share a common origin and which move in opposite directions from the origin. The initiation of replication unit synthesis also appears to be subject to temporal control. After initiation new daughter strands are synthesised, at least one strand is synthesised discontinuously as Okazaki fragments which are subsequently ligated. Nucleosome size may govern the initiation of Okazaki fragments whereas the temporal control of initiation on replication units and clusters may be controlled by higher orders of chromatin structure.

## 2.3 NUCLEOTIDE METABOLISM

The first step in DNA biosynthesis is the production of the nucleotide precursors (36). These are made available to the DNA synthesis machinery via two main pathways, the *de novo* biosynthesis pathways and the salvage pathways. The first type of pathway utilises ribose-5'-phosphate, certain amino acids, carbon dioxide and ammonia. These are combined in a series of successive reactions to form the free nucleotide-5'-monophosphates. There are two separate *de novo* pathways, one for purine synthesis and one for pyrimidine synthesis (Fig. 2.4 and Fig. 2.5).. In eukaryotes several of the enzymes for *de novo* synthesis are linked into a single multifunctional unit whereas in prokaryotes such as *E. coli* the enzymes can be extracted in soluble form.

The nucleotides formed in this way are ribonucleotides which then have to be converted to the deoxyribose form before they can participate in DNA synthesis. The enzyme responsible for this conversion is ribonucleoside diphosphate reductase. It catalyses the following reaction:

$$\text{riboNDP} + \text{reductant-(SH)}_2 \longrightarrow \text{deoxyriboNDP} + \text{reductant-(S-S)}$$

The cofactor supplying the enzyme with the pair of sulphydryl groups is thioredoxin. An alternative reductant found in *E. coli* mutants lacking thioredoxin is glutathione coupled to a protein glutaredoxin. This enzymes replaces the 2'-OH group of ribose with the hydrogen atom to give the deoxyribose sugar present in deoxyribonucleotides.

The nucleotide deoxythymidine, the nucleotide unique to DNA, is formed from dUMP by the enzyme thymidylate synthetase. This enzyme transfers a methyl group from the coenzyme methylene tetrahydrofolate to deoxyuridylate to give deoxythymidylate. The coenzyme is

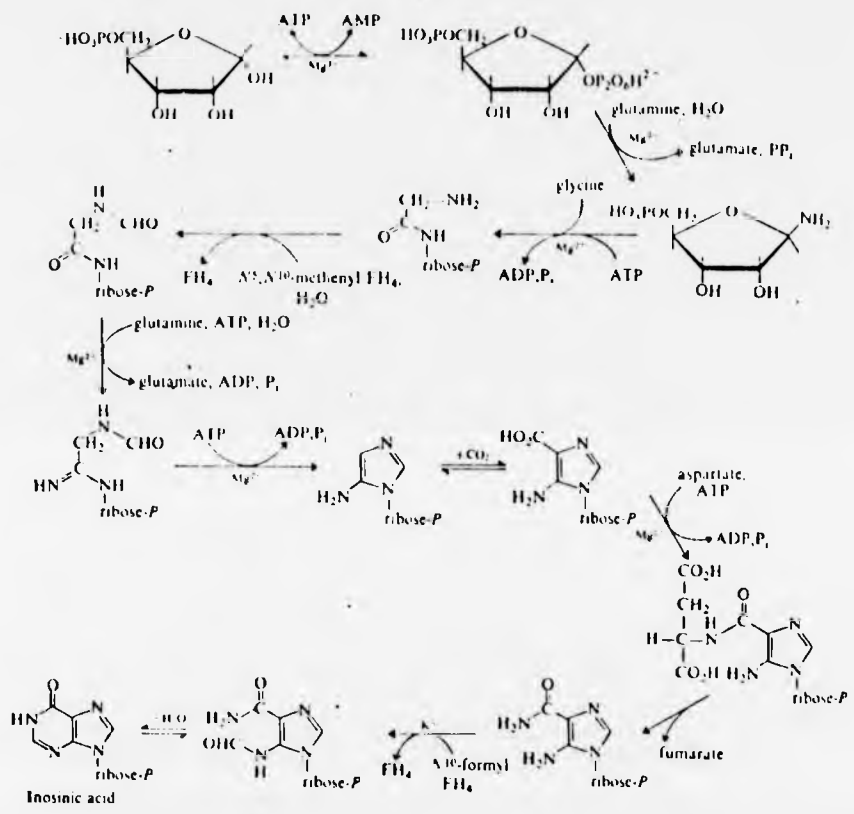



Fig 2.4a Purine biosynthesis: formation of inosinic acid

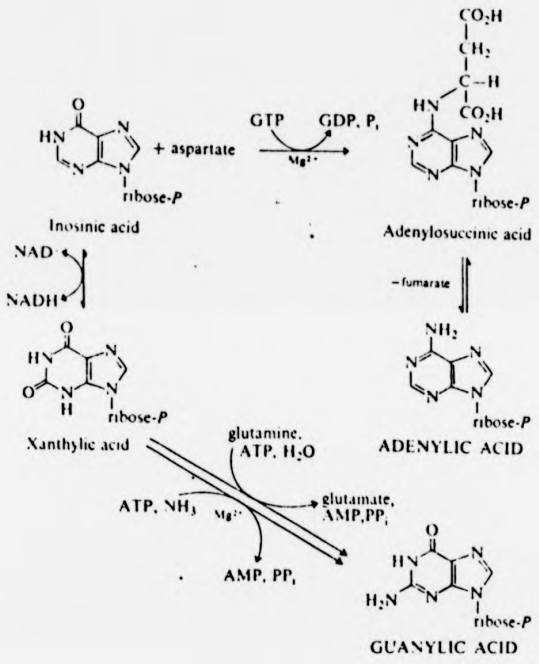



Fig 2.4b Purine biosynthesis: conversion of inosinic acid to adenylate and guanylate.

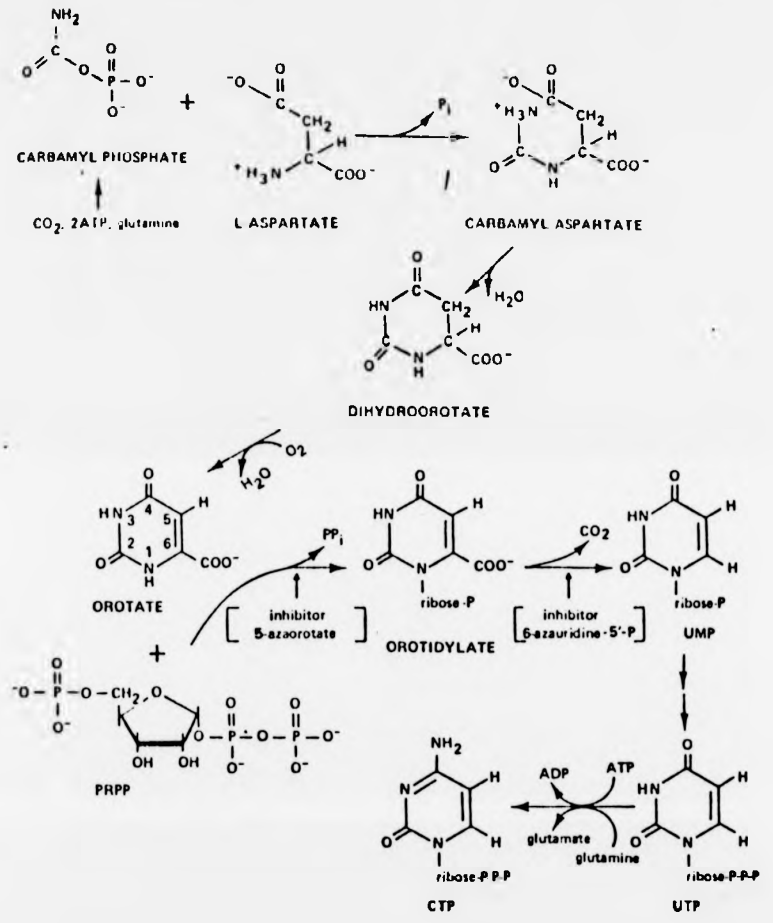
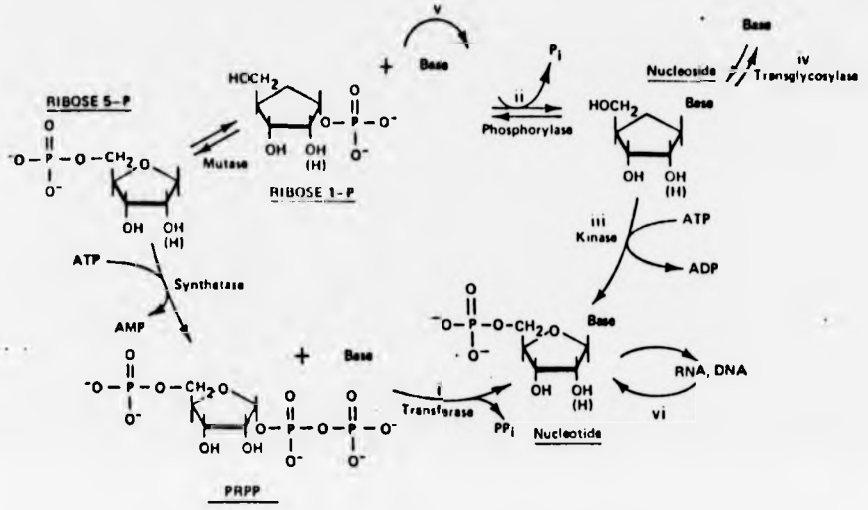



Fig 2.5 Pyrimidine biosynthesis




Principal pathways for salvage of bases and nucleosides.

Salvage pathways of nucleotide synthesis

| Pathway | Reaction | Enzymes |
|---|---|---|
| (i) Conversion of base to ribonucleotide | PRPP + base X ⇌ rNMP + $PP_i$ | phosphoribosyl transferase |
| (ii) Interconversion of bases and nucleosides | ribose 1-P + base N ⇌ nucleoside N + $P_i$<br>deoxyribose 1-P + base N ⇌ deoxynucleoside N + $P_i$ | nucleoside phosphorylase |
| (iii) Conversion of nucleoside to nucleotide | nucleoside N + ATP → NMP + ADP | nucleoside kinase |
| (iv) Base exchange into deoxynucleosides | base X + deoxynucleoside Y ⇌ base Y + deoxynucleoside X | nucleoside transglycosylases |
| (v) Interconversion by base alterations | adenine → hypoxanthine<br>cytosine → uracil | deaminases |
| (vi) Reutilization of nucleotides | rNMP → rNDP; dNMP → dNDP | nucleoside monophosphate kinase |
| | rNDP ⇌ rNTP; dNDP → dNTP | nucleoside diphosphate kinase |

Fig 2.6 The salvage pathways of nucleotide metabolism

regenerated by the enzymes dihydrofolate reductase, which catalyses the formation of tetrahydrofolate, and serine hydroxymethyltransferase which converts tetrahydrofolate to methylene tetrahydrofolate. The source of dUMP is dUTP produced by the action of the enzyme dUTPase.

The salvage pathways use the free bases and nucleosides ultimately produced by the breakdown of nucleic acids to provide an alternative source of precursors which can supplement *de novo* synthesis. This is essential, where due to mutation or the inherent genetic make-up of the cell, certain enzymes in *de novo* synthesis are lacking. When such a situation exists in pathogens, but not in the host's metabolism, these differences can be exploited as a rationale for the use of certain chemotherapeutic agents. As well as providing precursors for DNA synthesis salvage pathways may also serve important disposal or detoxification functions and play an important role in the drug metabolism of nucleoside and base analogues used in chemotherapy. A list of the important salvage reactions is shown in Fig. 2.6.

The nucleoside-5'-monophosphates do not participate directly in DNA synthesis and have to be first converted to the 5'-triphosphates, the substrates for the DNA polymerase enzymes. The monophosphates are first converted to diphosphates by base specific kinases. These kinases will however act on either ribose or deoxyribose nucleotides. The general reaction is:

$$NMP + ATP \rightleftharpoons NDP + ADP$$

Diphosphate synthesis is favoured by the rapid regeneration of ATP by oxidative and substrate-level phosphorylation. The diphosphates are converted to triphosphates by ubiquitous, non-specific kinases. The enzymes show no preference for any particular base or sugar. This lack of specificity also applies to the donor triphosphate but this

is generally ATP.

The synthesis of DNA precursors is under strict regulatory control and nucleotide pool size plays an important role in the regulation of DNA synthesis (37). There are two important points of regulation in purine nucleotide synthesis. The initial commitment to purine synthesis is under feedback regulation. The end products of the pathway AMP, ADP, ATP and GMP, GDP and GTP inhibit the enzyme amidophosphoribosyl transferase which condenses PRPP with glutamine to give phospho-ribosylamine. The second point of regulation occurs where the pathway diverges to form either adenylate or guanylate from inosinate. This point is under feedback control from ATP and GTP. If GTP is in excess more adenylate is formed and vice versa. The enzyme aspartate transcarbamylase is the key controlling enzyme in pyrimidine biosynthesis in *E. coli*. This enzyme is controlled by feedback inhibition from CTP and feedback activation from ATP thus balancing the synthesis of purine and pyrimidine nucleotides with one another.

Another important regulatory site for the production of DNA precursors is the enzyme ribonucleoside diphosphate reductase. This enzyme is under strict allosteric control responding to the metabolic needs of the cell, ATP acts as a positive effector whereas dATP acts as a negative effector when binding at the overall activity sites. When ATP or dATP bind at the substrate specificity sites reduction of UDP and CDP is favoured. Binding of dTTP stimulates GDP reduction and binding of dGTP stimulates ADP reduction thus giving fine control over the availability of specific DNA precursors.

## 2.4 INITIATION OF DNA SYNTHESIS

Growing cells go through a cyclic sequence of events known as the cell cycle. These can be summarised as $G_1$ - the post mitotic phase, S - the DNA synthetic phase, $G_2$ - the post synthetic phase and M - the mitotic phase. The control of entry into S phase from $G_1$ is the first regulatory point for DNA synthesis and is hypothesised to be controlled by cytoplasmic factors. A requirement for protein synthesis for the entry into S phase has been demonstrated indicating that progression from $G_1$ to S phase is controlled by a regulatory protein synthesised during $G_1$ and which acts as a positive effector to de-repress DNA replication (38).

The initiation of synthesis of replication units during S phase also requires protein synthesis (39, 40, 41) and evidence points towards the existence of a DNA binding protein which stimulates DNA synthesis and probably acts as a helix-destabilising protein. One protein of this type is the T antigen produced by SV40 virus (32, 42). This protein is required for the initiation of viral DNA replication and for the induction of cellular DNA synthesis. Other DNA binding proteins have been discovered for other viruses.

In most cases eukaryotic DNA replication is initiated at an origin within the replication unit and proceeds bidirectionally by the movement of two replication forks towards two termini. Recent evidence has suggested that in some instances replication may proceed unidirectionally. Proteins are probably involved in regulation at this level as initiation of unidirectional replication is preferred when normal replication is impeded by protein synthesis inhibitors. Attempts have been made to identify the origin of replication. These origins usually have a superhelical conformation and perhaps have a specific base sequence. In general initiation sequences seem to be

located within larger DNA segments that contain palindromes or tandem repeats of unique DNA length. A 73 base pair palindrome has been identified using restriction enzyme analysis as the point of origin of SV40 virus chromosome replication (39).

There is indirect evidence for a role for RNA in the initiation of eukaryotic DNA synthesis (32, 38, 42). Short chain DNA intermediates have been found in cells infected with SV40 virus in human lymphocytes and a number of mammalian cell lines containing short pieces of RNA at the 5' end. These RNA pieces are usually 8-11 nucleotides long and linked by 3', 5' bonds to DNA. The 5' end of the RNA sequence is not unique but can consist of either adenine or guanine residues. Analysis of RNA-DNA junctions reveals that all four common ribonucleotides and deoxyribonucleotides are present with about the same frequency.

There is little direct evidence for RNA involvement in initiation. Work on an isolated HeLa cell nuclei system has however shown a necessity for RNA polymerase I or an RNA polymerase I type enzyme for initiation of DNA synthesis (43). There is also a requirement for ribonucleotide triphosphates as a substrate for this enzyme. It is also suggested that other protein factors are involved in initiating DNA synthesis in this system. These include a heat-labile factor and a factor that can be removed from the nuclei by salt extraction. A role for these proteins has not been suggested but their existence seems to fit the pattern already indicated for the initiation of replication.

## 2.5 ELONGATION

The elongation of replicating eukaryotic DNA, once initiation has occurred, takes place in three stages (42). The primary event is the formation of 4-7S single stranded DNA segments resulting from the initiation of replication units, these are the Okazaki fragments. The

secondary elongation process proceeds through two major size classes, a 6-26 S segment and a 20-100 S segment arising from the arrangement of replication units either singly or in clusters. The third stage is the formation of chromosomal DNA from the joining together of the replication units. Elongation of eukaryotic DNA, like prokaryotic DNA, is a semi-conservative process and proceeds in the 5' to 3' direction. However there is some debate as to whether or not the process is semi-discontinuous as described for *E. coli* or fully discontinuous, i.e. there is no leading or lagging strand but both are synthesised from short Okazaki fragments. Recent evidence is in favour of eukaryotic DNA synthesis being totally discontinuous (35, 42).

Like prokaryotic DNA synthesis the involvement of several different proteins has been indicated though, as yet, their functions are not so well defined (32, 44). There is evidence for a strand separating protein in eukaryotes that binds to single stranded DNA. There is also an unwinding enzyme or nicking-closing enzyme that can relax the superhelical DNA formed by strand separation. The major proteins involved in eukaryotic DNA synthesis and the most well characterised are the DNA polymerases (45, 46, 47).

There are three DNA polymerases in higher eukaryotes that have been characterised by their size, template specificity and drug sensitivity and have been designated $\alpha$, $\beta$ and $\gamma$ (48). The main polymerase involved in normal mammalian DNA replication is DNA polymerase $\alpha$. This enzyme, a large 5-8 S protein, is located in the nucleus (49) and is sensitive to aphidicolin, araCTP and araATP, and the sulphydryl reagent N-ethylmaleimide (47, 48). Polymerase $\alpha$ prefers RNA primed DNA as a template. Evidence that it is the major replicative enzyme first came from observations of the marked rise in levels of this enzyme during cell replication. It is this enzyme that

synthesises the Okazaki size fragments of DNA. It is generally thought that nucleosomes govern the size of Okazaki pieces but there is evidence that suggests it may be the DNA polymerase enzyme itself (50).

DNA polymerase β is a smaller, 3-4 S, protein and is resistant to N-ethylmaleimide, aphidicolin and araCTP (47, 51). It cannot use RNA primed DNA as a template but will utilise activated (nicked) DNA and needs free 3'-OH termini as primers. The mode of DNA chain elongation by this enzyme appears to be distributive i.e. the enzyme molecule might leave the template at the 3' end of the growing chain after polymerization of one or more nucleotides and then reassociate with the template primer in a random fashion. The suggested role for this enzyme is as a repair enzyme for damaged DNA.

DNA polymerase γ is predominantly an extranuclear enzyme and is found in organelles such as mitochondria and chloroplasts though it is found in low levels in the nucleus. Its properties are similar to those of DNA polymerase α with regards to inhibitor sensitivity (48, 51). Its mechanism of action may be different to that of the other DNA polymerases as a result of its specific role in organelle DNA synthesis. It is the enzyme involved in adenovirus replication and it is studies on this virus that indicate a strand displacement mechanism of DNA synthesis similar to the rolling circle mechanism in bacteriophages. This gives some indication as to its possible role in the nucleus, a strand displacement mechanism would allow for gene amplification of certain parts of the chromosome e.g. ribosomal genes.

After the addition of deoxynucleotides to the RNA primer by DNA polymerase α the RNA primer is removed by an RNAase like enzyme called RNAaseH (42). This is followed by DNA gap filling. This is carried out by DNA polymerase α rather than by another polymerase as in *E. coli*. The Okazaki fragments when fully formed are joined

together by an ATP dependent ligase enzyme.

There are no exonuclease activities associated with the α or β enzymes as there is with the prokaryotic polymerases so there is no obvious proof reading mechanism in eukaryotes of the type found in prokaryotes. This implies that the high fidelity of DNA replication relies on the ability of the enzyme to copy the template accurately. A separate exonuclease activity has been found associated with DNA polymerase β and this may participate in repair.

Several forms of DNA polymerase α have been found, varying in their size. In rabbit bone marrow these have been called $\alpha_1$, $\alpha_2$ and δ (52). The two α polymerases have molecular weights 215,000 and 100,000 respectively and δ has a molecular weight of 122,000. The DNA polymerase δ enzyme possesses 3' to 5' exonuclease activity so it is possible that this is the form of DNA polymerase α used in normal replication thus providing it with a possible proof-reading function.

## 2.6 TERMINATION

There are two possible mechanisms for the termination of replication unit synthesis (38, 42). Termination could take place when two replication forks from adjacent replication units meet or. . there could be definite termination sites coded by specific nucleotide sequences. Evidence against the existence of specific termini comes from DNA fibre autoradiography studies on SV40 and polyoma viruses. In such cases where there is defective DNA i.e. during replication of viral DNA, chromosomal translocations, inversions or deletions, normal termination has still been observed, such occurrences would be impossible if termination was controlled by specific sequences.

Contrary to this is the fact that there is precise temporal and

spatial regulation of initiation and termination on widely separated, individual and tandemly arranged replication units. Such regulated DNA replication implies a specific control of initiation and termination and in fact termination sequences have been identified in some viral DNA molecules. It is therefore possible that structurally and functionally similar initiation-termination sequences are present on adjoining replication units of chromosomal DNA.

## 2.7 CHROMATIN ASSEMBLY

DNA synthesis proceeds as a component of chromatin replication. Concurrent with DNA replication is the unwinding of nucleosomal DNA in front of the replication fork and reassembly of nucleosomes behind the replication fork. Nucleosomes are assembled after the ligation of Okazaki fragements into nascent DNA chains. As there are only small histone pools in eukaryotic cells, histone synthesis has to be tightly coupled to DNA synthesis, therefore if one process is inhibited the rate of synthesis of the other is decreased. Newly synthesised DNA is rapidly associated with the histones into core particles to form nascent chromatin. Histone octamer assembly is conservative so that during chromatin replication when nucleosome numbers double half of the nucleosomes are made up of old histones and half are made up of new histones. There is no mixing of old and new histones in a nucleosome octamer. The nucleosomes nearest the replication fork comprising the nascent chromatin are different to those in mature chromatin therefore newly synthesised nucleosomes must go through a maturation stage during chromatin assembly. This maturation process may involve stepwise addition of histones to nascent DNA and may also reflect chemical modification of the histones.

The mechanism by which newly synthesised and parental nucleosomes are distributed and packaged with nascent DNA during chromatin replication

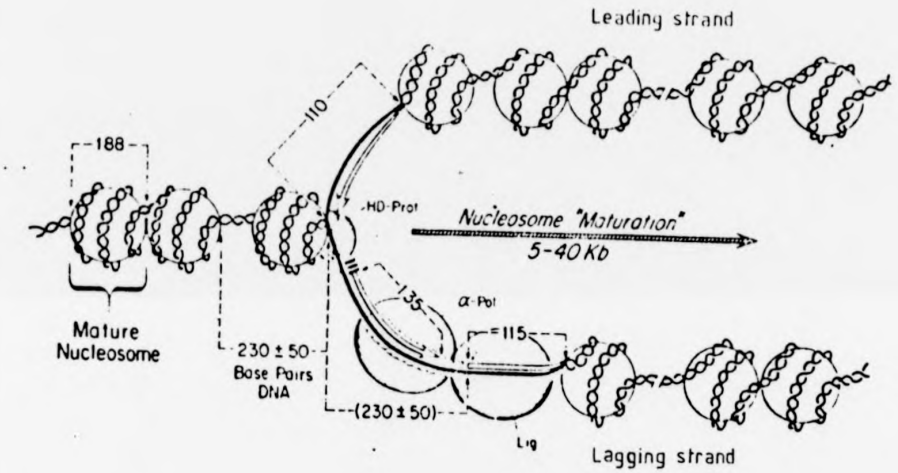



Fig 2.7 Replication fork of a eukaryotic chromosome.

HD helix destrabilising protein

α-pol DNA polymerase

lig DNA ligase

⇨ RNA primed Okazaki fragment

is not yet clear. There are two main models for chromatin assembly, the conservative chromatin replication model and the cooperative random model. Conservative chromatin assembly predicts that parental nucleosomes are to be found on only one daughter strand of DNA whilst the other consists entirely of newly made nucleosomes. The experimental evidence does not support this concept and the alternative hypothesis proposes that there is cooperative and random distribution of new and old nucleosomes between the daughter strands. This model requires that equal numbers of new and preformed nucleosomes be distributed to both growing DNA helices. The present experimental evidence supports the latter hypothesis for chromatin assembly (32, 42).

## 2.8 DNA AND CANCER

Induction of cancer can be correlated with changes in nucleotide sequence and gene expression caused by a carcinogen or mutagen. The repair of damaged DNA appears to be fundamental in preventing these genetic changes caused by local damage to the nuclear DNA and a great deal of work has been done to try to relate the repair of DNA lesions with carcinogenesis (53, 54, 55). The repair of damaged DNA involves a succession of reactions which include the excision of the abnormal base, removal of an adjacent sequence of DNA by exonuclease action, resynthesis of the removed section and finally ligation of the resynthesised DNA to the original molecule (53).

The possible connection between DNA repair and carcinogenesis was first suggested by studies on the hereditary disease, xeroderma pigmentosa (XP) which exhibits a genetic defect leading to a defect in the excision-repair system (19, 55). This disease is characterised by a sensitivity to ultraviolet light. Ultraviolet light causes the formation of dimers between adjacent thymidines causing local

distortion in the DNA double helix resulting in skin cancers. XP patients suffer an age-specific incidence of skin cancer that is several thousand times higher than normal and they frequently die of malignant melanoma.

One obvious contradiction within this theory is the fact that though the defect in DNA repair in XP patients is found in all tissues there is only an increased incidence in skin cancer but not in the incidence of other common fatal cancers. This suggests that most common fatal cancers are not caused by local DNA lesions of the kind that can be repaired by the DNA repair mechanism as was first proposed. An alternative origin of cancer must be found. In the case of Bloom's syndrome, an inherited disease that does appear to be associated with an increased incidence of common cancers, there is a high frequency of chromosomal aberrations and exchanges. Many leukaemias and lymphomas also show chromosomal aberrations, it therefore seems as if many cancers arise as a result of large chromosomal rearrangements or transpositions (19).

The regions of transposed DNA are known as transposons and their reshuffling within the DNA molecule is brought about by recombinational enzymes called transposases. This form of genetic rearrangement has been identified in lower eukaryotes and prokaryotes. It is not yet known if it plays a role in vertebrate development though it is known that antibody diversity is generated by specific transpositions during the differentiation of B lymphocytes. Though localised changes in DNA structure do give rise to some cancers as in the case of skin cancers it now seems as if many common fatal cancers arise as a result of genetic transpositions and that many carcinogens may be promoters of cancer rather than initiating cancers by producing localised DNA lesions.

# CHAPTER 3

## METABOLISM OF CYTOSINE ARABINOSIDE

### 3.1 METABOLISM OF CYTOSINE ARABINOSIDE

Cytosine arabinoside (1-β-D arabinofuranosyl cytosine) is a synthetic nucleoside which differs from the naturally occurring nucleosides, cytidine and deoxycytidine, in a substitution of the sugar arabinose for ribose or deoxyribose (56). It is one of the most effective and widely used antimetabolite drugs for the treatment of acute myeloid leukaemia giving complete remission rates of 25% when used alone and 50% or more when used in combination with other drugs such as daunorubicin, 6 thioguanine and alkylating agents (57, 58, 59, 60). Though the drug shows *in vitro* anti-viral activity, clinical tests have indicated that it is of little clinical use as an anti-viral agent (61, 62, 63). It also has marked immunosuppressive effects but is not used clinically as an immunosuppressant. The development of combination chemotherapy regimens of araC with other drugs has usually come about as a result of empirical clinical trials (64-68). It may be possible by studying the mechanism of action of this drug, its metabolism and the development of resistance to the drug by neoplastic cells to develop a biochemical rationale for combination chemotherapy. This chapter deals with a study of araC metabolism by some of the salvage enzymes involved in nucleotide metabolism, and the possibility of combination therapy of araC with other agents based on these studies is discussed.

The active form of the drug is the triphosphate but as this cannot cross the cell membrane the drug has to be administered as the nucleoside (69). AraC is rapidly transported into the cell by

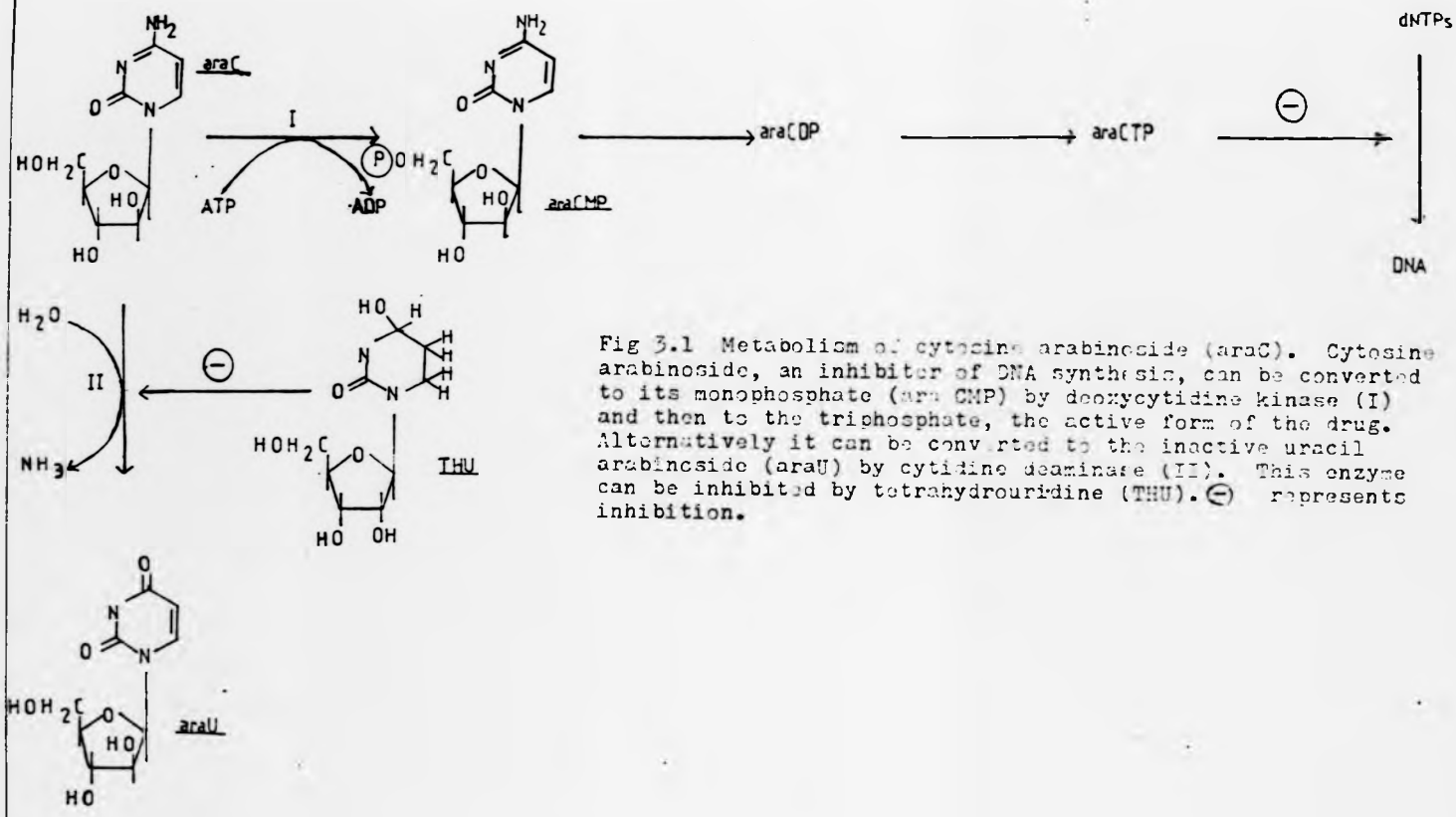



Fig 3.1 Metabolism of cytosine arabinoside (araC). Cytosine arabinoside, an inhibitor of DNA synthesis, can be converted to its monophosphate (araCMP) by deoxycytidine kinase (I) and then to the triphosphate, the active form of the drug. Alternatively it can be converted to the inactive uracil arabinoside (araU) by cytidine deaminase (II). This enzyme can be inhibited by tetrahydrouridine (THU). ⊖ represents inhibition.

a facilitated diffusion process similar to that used for transporting the naturally occurring nucleosides into the cell. Here the drug is phosphorylated by a series of kinase reactions to the triphosphate, the rate limiting step being the conversion to the monophosphate (70). Alternatively the drug can be inactivated by deamination to uracil arabinoside (71, 72). The therapeutic activity of the drug therefore depends on the balance that exists between the intracellular levels of these two enzymes.

In many instances tumour cells have been shown to develop resistance to the drug. There are a number of theoretically possible mechanisms for this resistance. These include (a) impaired transport into the cell, (b) impaired intracellular phosphorylation, (c) rapid deamination, (d) increased intracellular pools of the competing metabolite dCTP, (e) an altered form of the target enzyme, DNA polymerase, (f) variation in the plasma level of the intact drug, (g) competition between the normal metabolite and antimetabolite for the activating enzyme. The major reasons for drug resistance which have been suggested on the basis of experimental evidence are either low levels of the kinase responsible for converting the drug to the monophosphate (71), and/or high levels of the deaminase enzyme (72, 73) and/or increased intracellular pools of competing metabolites (74, 75). The activity of the drug is therefore dependent on the intracellular levels of the enzymes involved in its metabolism, primarily the kinase and deaminase. Recent work has shown that alterations in the regulation of the enzyme CTP synthetase may be involved in drug resistance (76). This enzyme is controlled by feedback inhibition from CTP. In cases when the enzyme is altered so that this feedback regulation is lost the CTP pool increases and this leads to an increase in dCTP levels. This results in a decreased sensitivity of the target cell to the drug due to the increased levels of the competing

nucleotide, dCTP.

Of the two major enzymes involved the kinase appears to be the most important one in determining the intracellular levels of araCTP. It is the level of the latter that determines the therapeutic efficacy of araC. This enzyme is the rate determining step in araC activation rather than transport of the nucleoside into the cell or the phosphorylation of the monophosphate to the di- and tri-phosphates. High levels of kinase have been found in leukocytes from patients with acute myeloid leukaemia (72). This correlates well with the fact that araC is most active against this type of leukaemia. Alternatively in cell lines tested for their *in vitro* response to araC those resistant to the drug are those with low levels of this enzyme.

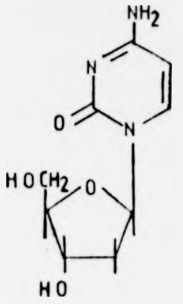
The enzyme responsible for the phosphorylation of araC to araCMP is the same enzyme that is responsible for the phosphorylation of deoxycytidine to dCMP (77, 78, 79). The two act as competitive inhibitors for each other thus suggesting that araC, biochemically speaking, is an analogue of deoxycytidine rather than cytidine. The enzyme is under allosteric control by dCTP, high levels of dCTP will inhibit the kinase. Deoxycytidine triphosphate also acts as a positive effector for the enzyme cytidylate deaminase. This converts dCMP to dUMP and can likewise deaminate araCMP thus acting against activation by the kinase and resulting in inactivation of the drug (80). Levels of dCTP can be controlled by inhibiting the enzyme ribonucleotide diphosphate reductase. Inhibition of this enzyme by inhibitors such as hydroxyurea will decrease the levels of dCTP. Other inhibitors acting at other stages of the salvage pathways such as alanosine and deazauridine will also decrease dCTP levels thus potentiating the phosphorylation of araC.

Cytidine deaminase, the enzyme responsible for the deamination

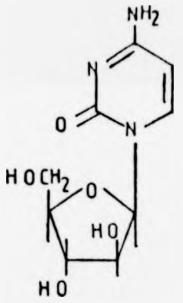
of araC to araU, is found in the liver, kidney, granulocytes, red blood cells and some tumours (81). The enzyme levels in normal granulocytes are higher than those in acute and chronic myeloid leukaemic cells, this may reflect an increase in enzyme levels with granulocyte maturation. The deaminase appears to confer resistance to araC in some cases of leukaemia. In a test carried out on patients, those that responded well to araC therapy had correspondingly low deaminase levels whereas those with high levels showed poor response to araC (73). In some cases deaminase levels increased during araC therapy and this corresponded with increased resistance to the drug implying that araC may induce the enzyme. Contrary to the clinical situation deaminase levels appear to have little effect on araC resistance in experimental tumour systems.

The enzyme has been isolated and well characterised in granulocytes especially with regard to its inhibition by the uridine analogue tetrahydrouridine (81). Tetrahydrouridine has been shown to be a potent inhibitor of cytidine deaminase (82). It is thought to be a transition state inhibitor resembling one of the intermediates in the reaction mechanism (81). THU has been shown to potentiate araC in some *in vitro* situations, e.g. in L1210 leukaemic mice and some murine tumours with high deaminase levels and sufficient kinase levels to activate araC (83, 84). Its possible role in combination chemotherapy with araC in the clinical situation has been demonstrated with some success especially with araC treatment of acute myeloid leukaemia though the tests are not altogether conclusive (85, 86). More recently other uridine analogues have been found that inhibit both cytidine and cytidylate deaminase, e.g. 3 deazauridine, which may prove to be effective in protecting araC from deamination (87).

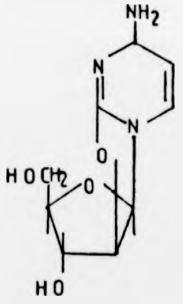
One alternative method of preventing deamination other than the



deoxycytidine



cytosine arabinoside



cyclocytidine

Fig 3.2 Deoxycytidine and analogues

use of inhibitors is to synthesise further analogues of araC that are resistant to deamination. Such analogues include a series of $N^4$ acyl derivatives with long chain saturated fatty acids (88, 89, 90) and conjugates of araC and steroids such as prednisolone and cortisol (91). Another, more generally used method, is to administer the drug as a continuous infusion so that the high levels will overcome the effect of deactivation. One method of enhancing this effect is to use the araC derivative cyclocytidine. This is slowly hydrolysed *in vivo* to give cytosine arabinoside thus achieving a sustained slow release of the active form of the drug (56).

## 3.2 LYMPHOBLASTOID CELL LINES

Transformed cells are cells derived from normal tissue that have undergone a stable hereditable change so that they can be continuously cultured *in vitro*. The two cell lines used here are both derived from lymphocytes. The major lymphoblastoid cell line that has been used is a B cell line transformed by the Epstein-Barr virus and derived from a Burkitt's-type lymphoma (20). Part of the EB virus genome is incorporated into the genetic material of the transformed cells, the cells then produce EBV directed antigens each of which serves a specific function in the replication of the infected cell and subsequent viral replication (92). The major antigens are the nuclear antigen (EBNA), the early and late membrane antigens (EMA and LMA), the early antigen complex (EA), viral capsid antigen (VCA) and the lymphocyte-detected membrane antigen (LYMA). EBNA is the only antigen present in all EBV transformed B cell lines and is necessary for the continued growth of the infected cells. MA, EA and VCA are only expressed in cell lines that produce EBV. The cell line grown in this laboratory, the Namalwa cell line, only produces EBNA and is therefore a non-producer cell line, i.e. it is not possible for this

particular cell line to produce new, infectious EB viruses thus making it an ideal cell line for use in the laboratory. This cell line is relatively easy to grow compared with many other cell lines and provides a useful model system for the study of immunosuppressive and anti-cancer drugs.

The other cell line used in these studies is a T cell line derived from an acute lymphocytic leukaemia (93). This lymphoblast cell line, the CCRF-CEM cell line, was originally isolated from the peripheral blood of a female child suffering from acute childhood leukaemia. This T cell line was used as part of a comparative study of cytosine arabinoside metabolism.

## 3.3 TETRAHYMENA PYRIFORMIS

*Tetrahymena pyriformis* is a ciliated protozoan normally found inhabiting fresh water environments (94). It is pyriform or pear shaped with an average cell size of 50 x 30 μm dependent upon environmental conditions. *Tetrahymena* possess typical eukaryotic organelles such as mitochondria, rough and smooth endoplasmic reticulum, nucleus and lysosomes. They also contain food vacuoles and microbodies called peroxisomes, responsible for oxidising α-hydroxy and α amino acids and metabolising lipids to glycogen via the glyoxylate cycle, which are not found in higher eukaryotes. Many strains of *Tetrahymena* also contain a diploid micronucleus. This plays a role in the protozoans sexual reproductive cycle. Those strains that are amicronucleate reproduce only by vegetative, mitotic growth. The macronucleus is polyploid carrying several copies of the genome. It is the genetic information carried in the macronucleus that determines the phenotype of the protozoan.

The strain used in the work described in this chapter is the

amicronucleate strain, *T. pyriformis w*. This strain, which is only capable of vegetative growth, can be grown easily and rapidly in the laboratory in both defined and complex media (95). If necessary cultures can be induced to divide synchronously making it a suitable organism for cell cycle studies. As it possesses many typical eukaryotic characteristics it can be used as a model system for the study of eukaryotic metabolism both in whole cell and cell free systems. It has been used extensively in this laboratory for studying DNA, RNA and cyclic nucleotide metabolism. Because it has been well characterised biochemically it was used as a control system in a comparative study of cytosine arabinoside metabolism.

## 3.4 MATERIALS AND METHODS

### 3.4.1 Materials

Namalwa and CCRF-CEM lymphoblast cell lines were obtained from the Department of Biological Sciences, University of Warwick, *T. pyriformis* strain L1630/IW was obtained from the Culture Centre of algae and protozoa, Cambridge. RPMI-1640 medium and new born calf serum were obtained from Flow Laboratories and proteose peptone and yeast extract from DIFCO Laboratories. Glutamine, penicillin, streptomycin and cytosine arabinoside were obtained from Sigma Chemical Company, trypan blue from BDH Chemicals and tetrahydrouridine and calf thymus DNA from Calbiochem. Prefilled DOWEX50 columns were obtained from Biorad Laboratories, PEI-F cellulose thin layer chromatography plates from Merck and DEAE cellulose DE81 chromatography paper and Whatman No. 1 chromatography paper from Whatman Ltd. [$^{3}$H-methyl] thymidine and 5-$^{3}$H cytosine β-D arabinoside were obtained from the Radiochemical Centre, Amersham.

### 3.4.2 Culture of lymphoblastoid cell lines

Namalwa and CCRF-CEM cells were grown under aseptic conditions at 37°C in Hepes buffered RPMI-1640 medium supplemented with 10% newborn calf serum, L-glutamine (300 mg/l), penicillin (100 u/ml) and streptomycin (100 µg/ml). Cultures of 50 ml or more were grown in 2 l roller bottles, smaller volumes were grown in stationary cultures. The cells were grown to a density of $1.2 \times 10^6$ cells/ml and then passaged to give a final cell concentration of $0.6 \times 10^6$ cells/ml, the average doubling time being three days. At regular intervals of about 10-14 days the cells were harvested by centrifugation at 100 g, 5 minutes, 18-20°C and resuspended completely in fresh medium. Regular supplementation with L-glutamine was also essential as it is rapidly degraded under normal culture conditions. The cells were counted and their viability determined using the trypan blue exclusion test. A 0.5 ml aliquot of cells was diluted with 0.5 mls 0.4% trypan blue in 0.9% saline and then counted in a Neubauer haemocytometer. Dead cells are stained blue whereas live cells remain unstained due to their ability to pump out the dye.

### 3.4.3 Culture of *T. pyrifomis*

*Tetrahymena pyriformis* was grown under aseptic conditions in 2% proteose peptone, 0.1% yeast extract, 0.5% glucose and 5 µg/ml ferric chloride all made up in tap water. 50 ml static cultures were grown in 250 ml conical flasks in the dark at room temperature and subcultured weekly by addition of a 0.2 ml inoculum of old cultures into 50 ml fresh medium.

The stock cultures were used to inoculate 400 ml cultures in 2 l conical flasks, the desired volume of cell inoculum was calculated from the following equation:

$$v = \frac{y.V}{x.2^{(t/3-1)}}$$

v = volume of inoculum required (ml)

y = desired final cell concentration in cells/ml

V = final culture volume (400 ml)

x = cell concentration of stock culture

t = time in hours from time of inoculation to the time when the cells are required.

The 400 ml cultures were incubated at 28°C in an orbital shaker operating at a rate of 150 oscillations/minute. Under these conditions the average doubling time for the cells was three hours. The cells were counted by fixing an aliquot of cells with an equal volume of 20% formalin in 0.01 M phosphate buffer pH 7 and then counting in a Neubauer haemocytometer.

### 3.4.4 Measurement of $^{3}$H-thymidine incorporation by lymphoblasts

1 ml cultures of Namalwa cells, $10^6$ cells/ml, were incubated in 10 ml Sterilin tissue culture tubes at 37°C for four hours with 5 µCi [$^{3}$H-methyl] thymidine, sp. act. 47 Ci/mmol. Cytosine arabinoside and tetrahydrouridine were both dissolved in PBS and added to the cultures to give the required final concentration. After incubation the cultures were diluted with 5 mls cold PBS and centrifuged at 200 g, 10 minutes, 0-4°C. The supernatant was discarded and the precipitate was resuspended in 200 µl calf thymus DNA, 1 mg/ml in 5 mM NaOH, and 500 µl of cold 5% TCA added. The acid insoluble precipitate was collected by centrifugation at 200 g, 5 minutes, 0-4°C and washed in the same way with a further 500 µl cold 5% TCA. The precipitate was

then dissolved in 500 µl 0.5 M NaOH and 200 µl aliquots removed and added to 8 mls scintillation fluid (Toluene 600 mls: ethoxyethanol 400 mls, PPO 4 g/l, POPOP 0.2 g/l). The samples were left overnight to remove any chemiluminescence caused by the sodium hydroxide. The radioactivity of the samples was then counted on a Packard Tri-Carb 2425 liquid scintillation spectrometer. Results are shown as the percentage of maximum thymidine incorporation into acid precipitable material.

### 3.4.5 Identification of araC metabolites

1 ml cultures of Namalwa and CCRF-CEM cells, $10^6$ cell/ml, were incubated with 5 µCi 5-$^3$H cytosine β-D arabinoside, sp. act. 26 Ci/mmol, both in the presence and absence of $10^{-4}$ M THU for 0, 0.5, 1, 2, 3 and 4 hours. At the end of the incubation period the cultures were washed in 5 mls cold PBS and the precipitate resuspended in 500 µl cold 5% TCA. The acid insoluble precipitate was removed by centrifugation at 200 g, 5 minutes, 0-4$^{\circ}$C and 15 µl of the supernatant applied to prepared PEI-F cellulose thin layer chromatography plates (96). For *T. pyriformis* 30 ml cultures of log phase cells, 2-4 x $10^5$ cell/ml, were incubated at 28$^{\circ}$C in a shaking water bath with 20 µCi $^3$H-cytosine arabinoside with or without $10^{-4}$ M THU. 1 ml aliquots were removed at 0, 0.5, 1, 2, 3 and 4 hours and treated as described for the lymphoblastoid cells.

The plates were prepared by first running the plates in 10% sodium chloride for 5 cms and then transferring to distilled water without drying and allowed to run until the solvent front ran off the edge of the plate. The plates were then dried for two hours in air and run again in distilled water until the solvent front ran off the edge. The plates were then left to dry overnight in air.

The plates, containing the samples, were then placed in distilled water until the solvent front had risen 5 cms. They were then transferred without drying to an acid solvent of 1 M lithium chloride, 1 M formic acid where they were run for a further 5 cms. The plates were then removed and dried in air.

After drying the plates were cut into 1 cm squares and placed in scintillation vials with 0.5 ml 1 M LiCl, 1 M HCOOH and left at room temperature for two hours. Toluene, ethoxyethanol, PPO, POPOP scintillant was then added (12 mls) and radioactivity counted as previously described. The metabolites were identified by running standards simultaneously with the samples. The position of the standards on the TLC plates was determined by their ability to fluoresce under UV light of wavelength 254 nm.

### 3.4.6 AraC deaminase assay

Cell homogenates of both lymphoblastoid cell lines and *T. pyriformis* were prepared in 0.001 M EDTA, 0.002 M DTT, 0.05 M Tris-HCl pH 8 buffer. Lymphoblastoid cells, $10^6$ cells/ml, and *T. pyriformis*, 2-4 x $10^5$ cells/ml, were harvested as previously described, washed in cold PBS, and the pellet resuspended in the homogenising buffer. The cells were then homogenised in a motor driven Potter-Elvejhem homogeniser at 4°C, the degree of cell breakage was monitored using a light microscope. The ability of these cell homogenates to deaminate araC to araU was assayed using the method described below. As the substrate used in this assay was araC the enzyme is described as araC deaminase though the enzyme is probably isofunctional with cytidine deaminase.

The assay mixture contained 100 µl cell homogenate, 50 µl $^3$H-araC, 5 µCi/ml at a final concentration of 5 x $10^{-5}$ M, and 100 µl homogenising buffer or 75 µl buffer plus 25 µl tetrahydrouridine to give the

required final concentration. The assay mixture was incubated at 37°C for the lymphoblast homogenates and 28°C for the *Tetrahymena* homogenates. The incubation was stopped by the addition of 250 µl 3 M HCl and the resultant precipitate removed by centrifugation for 10 minutes in a BTL microangle bench centrifuge. The supernatants were then placed on top of 4 cm pre-filled columns packed with DOWEX 50W-X8 200-400 (97) and allowed to sink into the column bed. The columns were then eluted first with 2.5 ml of the homogenising buffer to remove the arabinosyl uridine formed in the reaction. The araC bound to the columns was then removed by eluting with 2.5 ml 3 M ammonia. The columns were then washed several times with distilled water to prepare them for further use. 200 µl aliquots were removed from the eluates and added to 8 mls of Toluene, ethoxyethanol, PPO, POPOP scintillant and radioactivity determined as previously described.

The compounds eluted from the columns were identified using descending paper chromatography. Samples from selected eluates were spotted on to Whatman No. 1 chromatography paper and run for 10 cms in isobutyric acid, ammonia, water (66:1:33). The chromatogram was then dried in air and cut into 1 cm squares and placed in scintillation vials with 0.5 mls 3 M HCl and left at room temperature for two hours. Toluene, ethoxyethanol, PPO, POPOP scintillant (12 mls) was then added and the radioactivity of the samples determined. The products of the reaction were identified by running standards simultaneously with the samples on the chromatogram. All protein determinations were carried out by the method according to Lowry (98) using bovine serum albumin as a standard.

### 3.4.7 AraC kinase assay

Cell homogenates prepared as described in 3.4.6 were assayed for their ability to phosphorylate araC to araCMP. As in the case of the deaminase assay the substrate used was araC therefore the enzyme is described as araC kinase though it is probably iso-functional with deoxycytidine kinase. The assay incubation mixture contained 100 µl cell homogenate, 100 µl assay cocktail and 50 µl $^{3}$H-araC, 5 µCi/ml at a final concentration of $5 \times 10^{-5}$ M. The assay mixture was incubated at 37°C for the lymphoblast homogenates and 28°C for the *Tetrahymena* homogenates. The assay cocktail was made up of equal volumes of the following components plus two volumes of distilled water:

| | | |
|---|---|---|
| ATP | 0.14 M | Final concentration 0.01 M |
| $MgCl_2$ | 0.07 M | Final concentration 0.005 M |
| 2 Mercaptoethanol | 0.14 M | Final concentration 0.01 M |
| Sodium fluoride | 0.07 M | Final concentration 0.05 M |

Homogenising buffer (Tris HCl pH 8 0.05 M, 0.001 M EDTA, 0.002 M DTT)

The assay mixture was incubated for up to thirty minutes and stopped by heating for three minutes in a boiling water bath. The resultant precipitate was removed by centrifugation for ten minutes in a BTL micro-angle bench centrifuge. 25 µl samples were removed from the supernatant and pipetted on to DEAE (DE81) 1.5 cm filter squares (71, 98). The filter squares were then washed for 10 minutes in 500 ml 0.001 M ammonium formate followed by 45 minute and 20 minute washes in 250-500 ml distilled water leaving radioactive araCMP bound to the filter squares. The filter squares were then placed in scintillation vials with 1 ml 0.5 M NaCl in 1 M HCl for 10 minutes with occasional shaking. Scintillation

fluid (10 mls of Toluene 50% v/v, Triton X-100 50% v/v, PPO 5 g/l, POPOP 0.2 g/l) was then added and the radioactivity of the samples determined. The products of the assay were characterised using thin layer chromatography on PEI-F cellulose plates using the conditions described in 3.4.4 (A.M. Coles, Ph.D. dissertation, University of Warwick, 1981).

## 3.5 RESULTS

### 3.5.1 The effect of cytosine arabinoside and tetrahydrouridine on $^{3}$H-thymidine incorporation by Namalwa cells

The incorporation of [$^{3}$H-methyl] thymidine into acid preciptable material was used as a measure of DNA synthesis. Cytosine arabinoside, a known inhibitor of DNA synthesis (see Chapter 4), inhibited the incorporation of $^{3}$H-thymidine into acid precipitable material by Namalwa cells, the greater the concentration of araC the greater the degree of inhibition until maximum inhibition was obtained. Increasing concentrations of tetrahydrouridine had a negligible effect on $^{3}$H-thymidine incorporation of Namalwa cells. THU should increase the inhibitory effect of araC when used in combination with araC if the latter is inactivated by deamination. The effect of increasing concentrations of THU while maintaining araC concentrations constant at $5 \times 10^{-9}$ M was investigated. This concentration of araC gave approximately 50% inhibition of thymidine incorporation by Namalwa cells in the absence of THU. THU however had a negligible effect on araC inhibition at this concentration. It remained at approximately 50% with all the THU concentrations used.

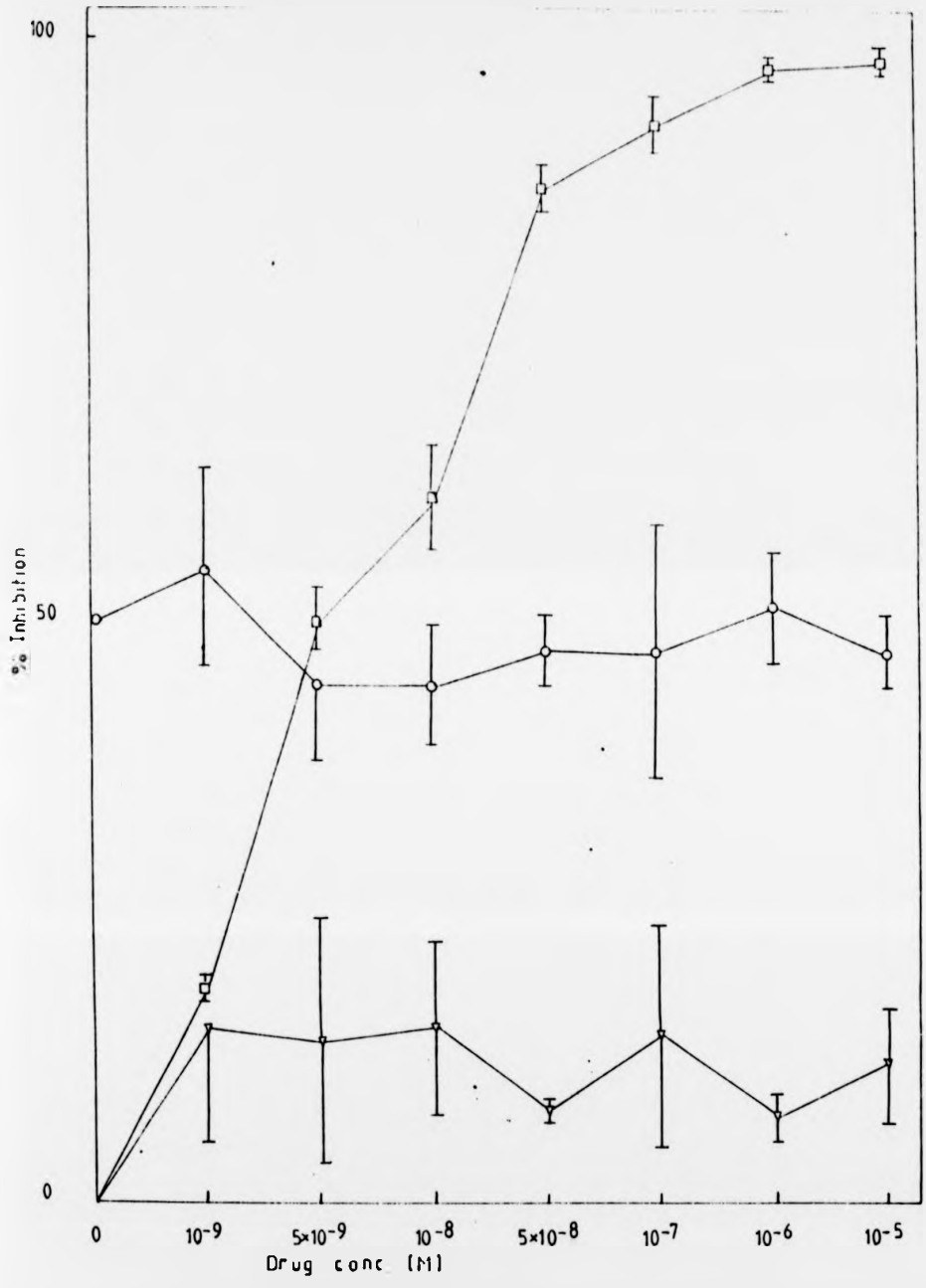



Fig 3.3 The inhibition of $^{3}$H-thymidine uptake in Namalwa cells by (□) cytosine arabinoside, (▽) tetrahydrouridine and (○) tetrahydrouridine in the presence of $5\times10^{-9}$M cytosine arabinoside. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E., n=3.

### 3.5.2 The metabolism of cytosine arabinoside by *T. pyriformis w*

The products from the incubation of *T. pyriformis* over a four hour period with $^{3}$H-cytosine arabinoside are shown on the thin-layer chromatogram profiles (Figs. 3.4, 3.5). Within the first hour there was rapid conversion of araC to araU. There was little phosphorylated product formed from either of the two nucleosides within that period. There was an increased assimilation of araC after that time though this was still accompanied by rapid deamination. After the full four hour period there was some increase in the amounts of the phosphates formed though araUTP predominated over araCTP. These results indicate the presence of a very active deaminase enzyme but very low kinase activity.

The incubation procedure was repeated in the presence of $10^{-4}$ M tetrahydrouridine. The presence of the uridine analogue lead to a marked decrease in the araU levels within the first hour. There were few phosphorylated products formed within that period. As the incubation continued there was an increase in the level of araCTP and a steady uptake of araC. There was no formation of araU or araUTP. It therefore appears as if the THU completely inhibited the deamination of araC. There was no increase in the degree of phosphorylation but the product in this case was only araCTP, rather than a mixture of araUTP and araCTP, resulting in increased intracellular levels of the drug.

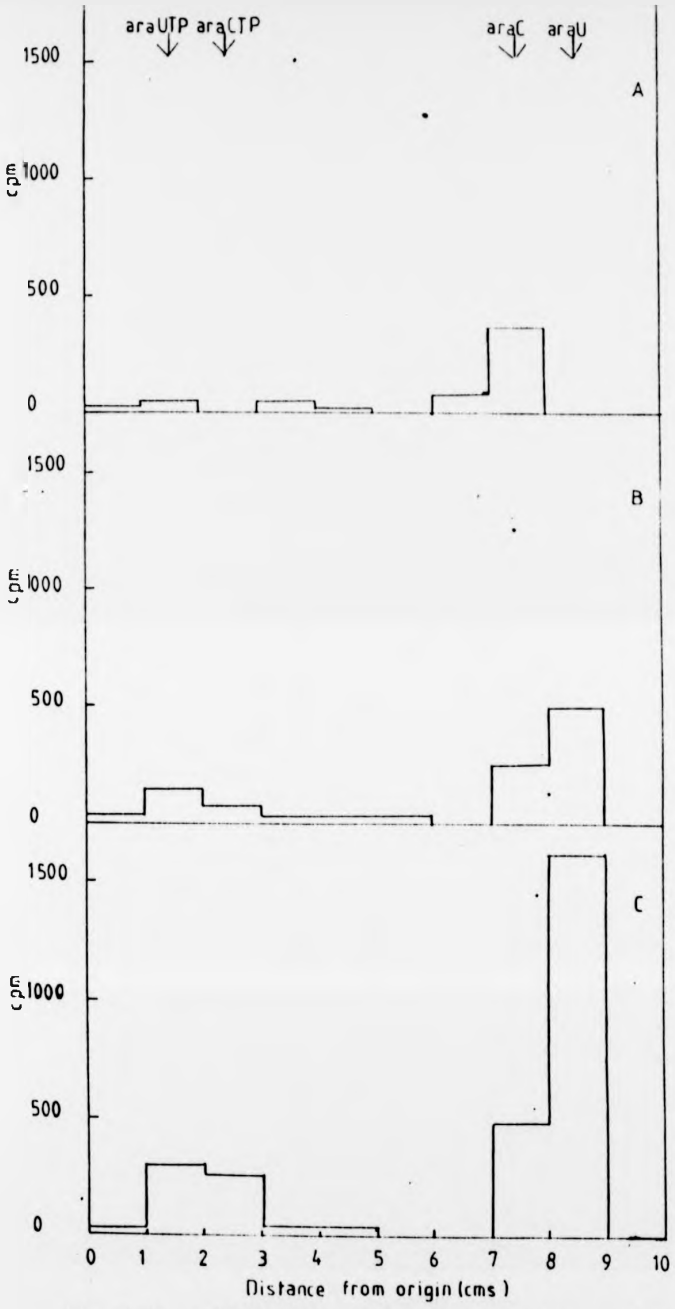



Fig 3.4a Identification of the products of cytosine arabinoside metabolism by T. pyriformis. 30ml cultures of the protozoan were incubated with $^{3}$H-araC and 1ml samples removed at regular time intervals and the tritiated products identified using PEI - cellulose thin layer chromatography as described in 3.4.5. The profiles shown are for samples removed at (A) 2mins, (B)30mins and (C) 1hour.

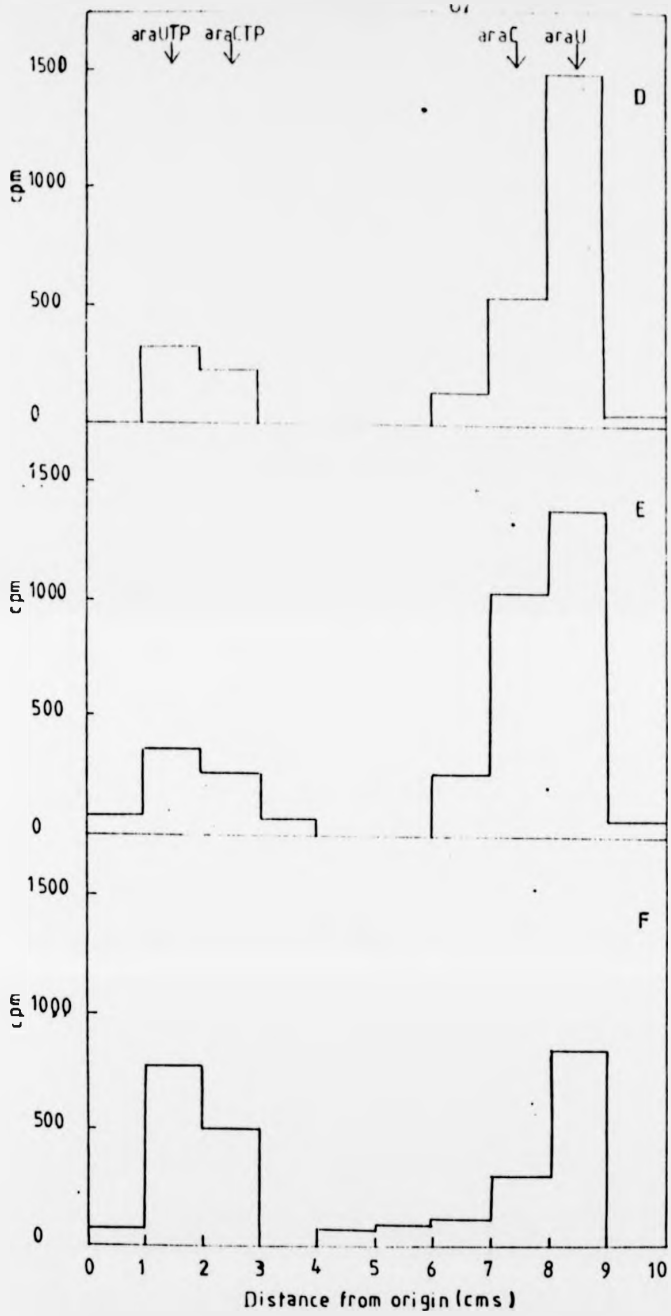



Fig 3.4b Identification of the products of cytosine arabinoside metabolism by T. pyriformis. as described in 3.4.5. The thin layer chromatography profiles shown are for samples removed at (D) 2hours, (E) 3hours and (F) 4hours.

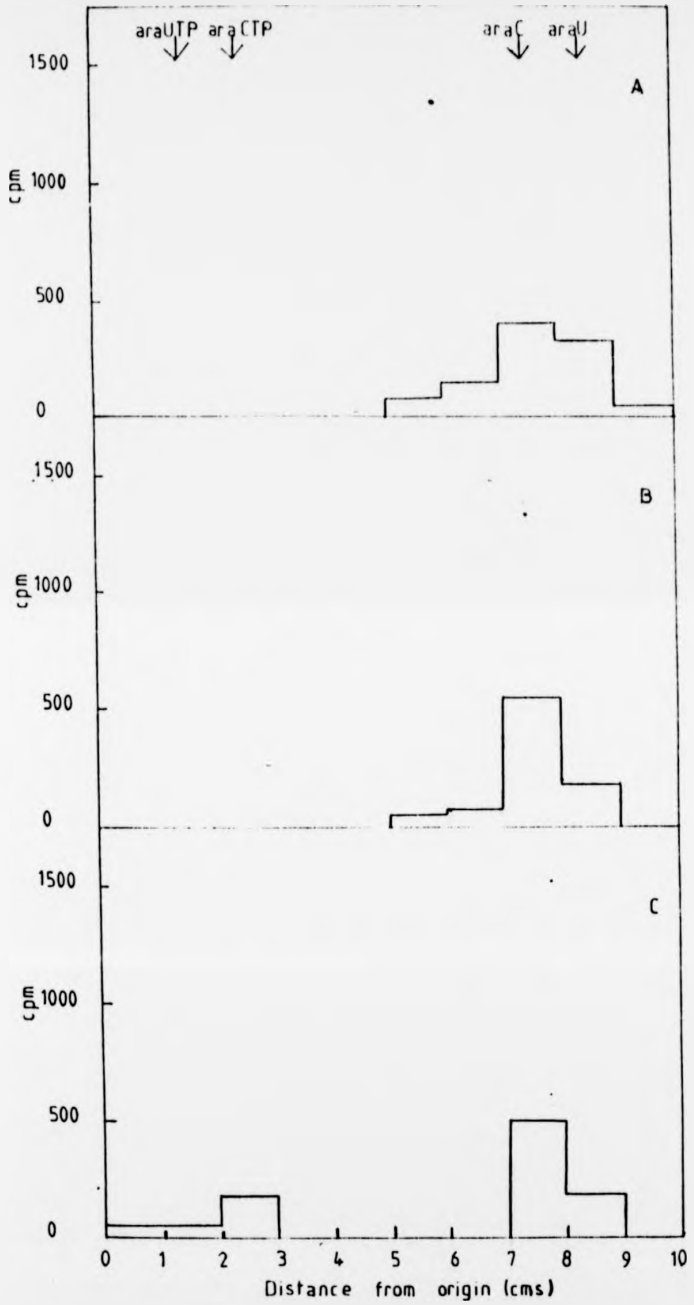



Fig 3.5a The effect of tetrahydrouridine on the metabolism of cytosine arabinoside by T.pyriformis. 30ml cultures of the protozoan were incubated with $^{3}$H-araC in the presence of $10^{-4}$M tetrahydrouridine. 1ml samples were removed at regular intervals and the tritiated products identified using PEI-cellulose thin layer chromatography as described in 3.4.5. The profiles shown are for samples removed at (A)0mins (B) 30mins and (C) 1hour.

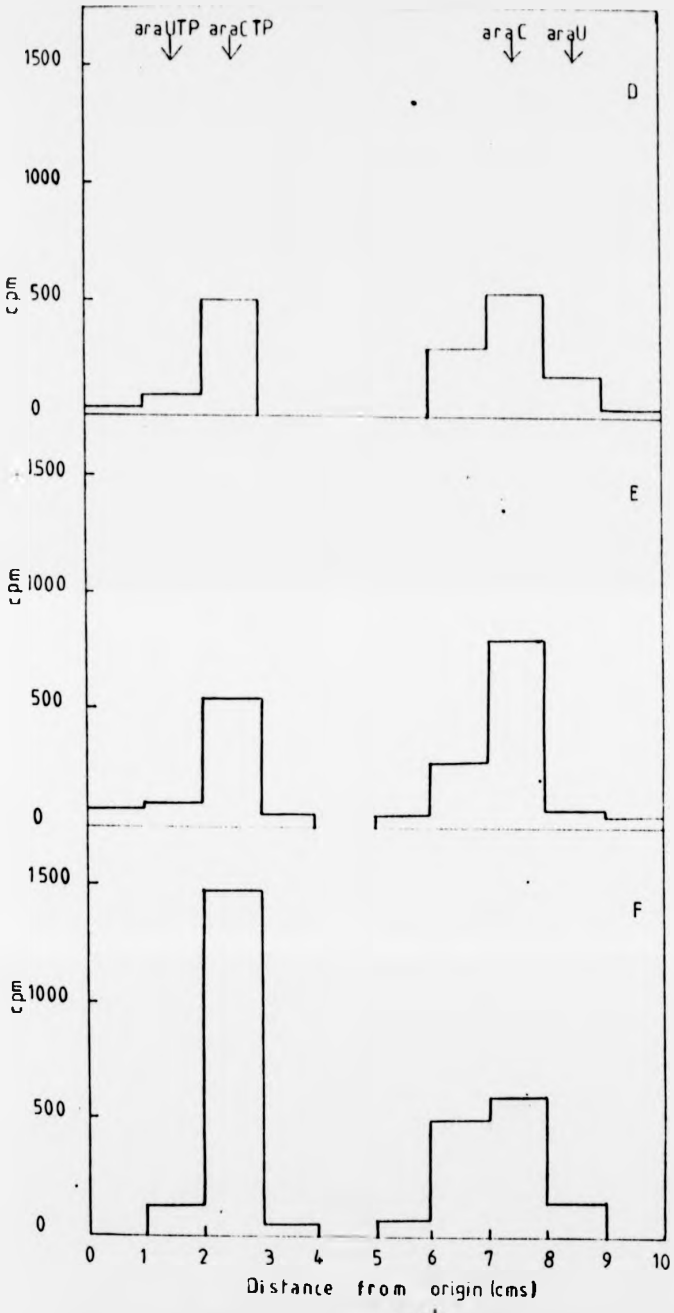



Fig 3.5b The effect of $10^{-4}$M tetrahydrouridine on the metabolism of cytosine arabinoside by T. pyriformis as described in 3.4.5. The thin layer chromatography profiles shown are for samples removed at (D) 2hours, (E) 3hours and (F) 4hours.

### 3.5.3 Metabolism of cytosine arabinoside by lymphoblastoid cell lines

The thin-layer chromatogram profile produced by the incubation of $^{3}$H-araC with Namalwa cells shows a steady increase in araCTP levels throughout a four hour period suggesting that the araC was phosphorylated rapidly as it entered the cell. There was no indication of the formation of araU or its phosphorylated derivatives suggesting the absence of any araC deaminase activity (see Fig 3.6)

The incubation was repeated in the presence of $10^{-4}$ M THU. This had a negligible effect on the metabolism of the $^{3}$H-araC giving a thin-layer chromatogram profile of araC metabolites similar to that produced without THU. The same procedure was also carried out using the CCRF-CEM cell line both in the presence and absence of THU. These incubation procedures gave similar results to those obtained from the incubation of $^{3}$H-araC with Namalwa cells. The profile shown for the incubation of $^{3}$H-araC with Namalwa cells is representative of similar profiles obtained from the series of experiments described above.

### 3.5.4 Characterisation of the araC deaminase assay using *T. pyriformis*

The principle of the assay relies upon the ion exchange properties of the DOWEX 50 columns. The sulphonate groups on the column matrix bind the amino group of cytosine arabinoside allowing the deaminated uracil arabinoside to pass through. The cytosine arabinoside is then eluted from the column by washing with 3 M ammonia solution which displaces the bound araC. In the first characterisation experiment the *T. pyriformis* cell homogenate was incubated with the $^{3}$H-araC as described in "Materials and Methods"

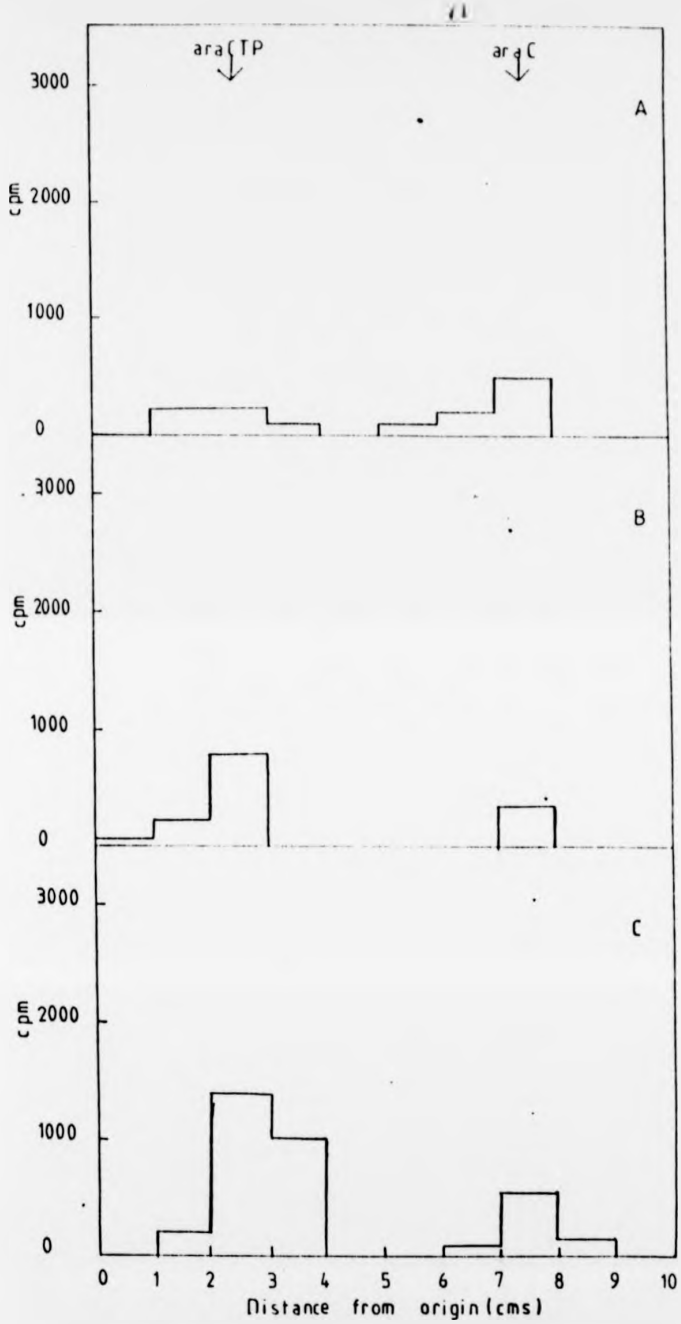



Fig 3.6a Identification of the products of cytosine arabinoside metabolism by Namalwa cells. 1ml cultures of lymphoblastoid cells were incubated with $^{3}$H-araC for increasing time intervals and the tritiated products identified using PEI-cellulose thin layer chromatography as described in 3.4.5. The profiles shown are for cultures incubated for (A) 0mins (B) 30mins (C) 1hour.

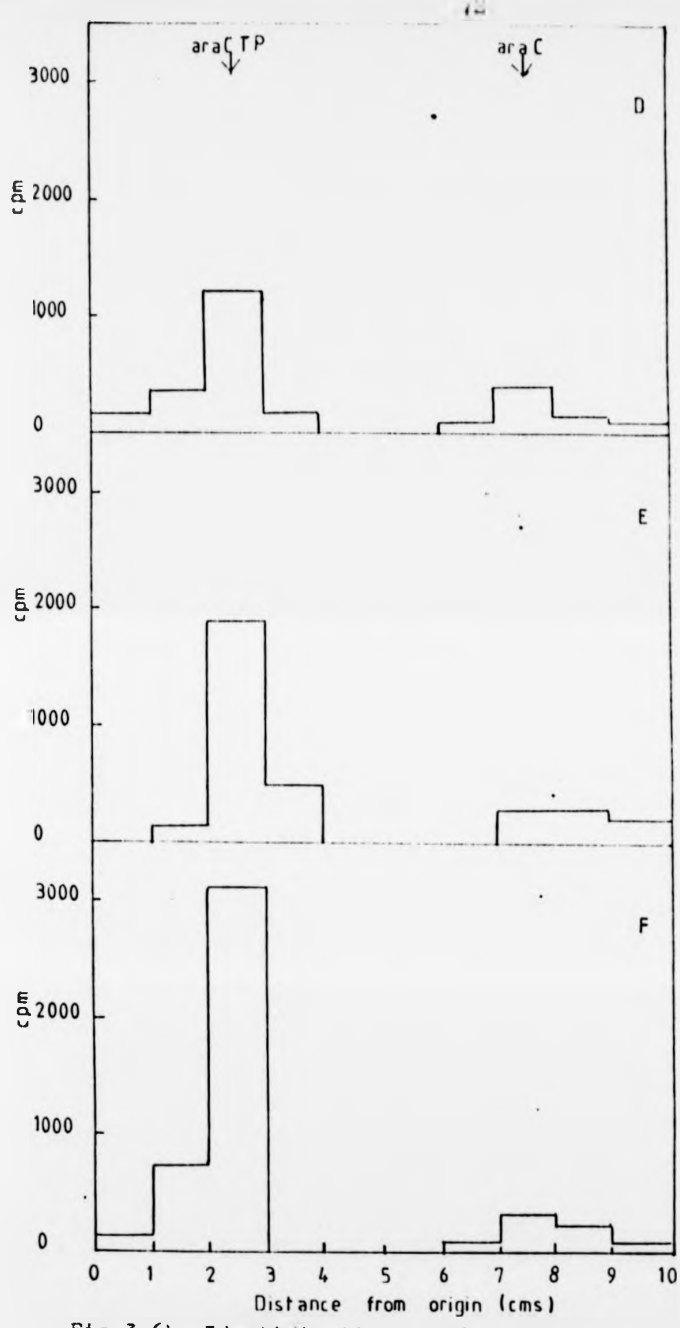



Fig 3.6b Identification of the products of cytosine arabinoside metabolism by Namalwa cells as described in 3.4.5. The thin layer chromatography profiles shown are for cultures incubated for (D) 2hours (E) 3hours and (F) 4hours.

for 0, 15, 30, 45 and 60 minutes. The incubation mixture, after treatment with 3 M HCl was carefully pipetted on to the columns and eluted as described. Samples were taken from the buffer and ammonia washes for radioactivity determinations. As can be seen in Figure 3.7 the radioactivity in the buffer wash increased with a corresponding decrease in the radioactivity in the ammonia wash. The total amount of radioactivity remained constant throughout.

Samples were taken from both washes from the 0 and 60 minute incubations and spotted on to Whatman No. 1 chromatography paper as described in "Materials and Methods" in order to identify the radioactive products in each wash at the beginning and end of the assay. At 0 minutes there was no radioactive product in the buffer wash as would be expected from Fig. 3.7. In the ammonia wash the only radioactive compound was the substrate, $^{3}$H-araC. After a 60 minute incubation there was a large amount of $^{3}$H-araU in the buffer wash and again no $^{3}$H-araC. There was some residual $^{3}$H-araC in the ammonia wash.

These two results indicate the validity of the method as an assay for araC deaminase. Both sets of results are representative results from a series of similar experiments.

A time course for the assay was performed in order to find out over what period the reaction was linear with time so that the specific activity could be calculated and also so that suitable conditions could be determined for assays on lymphoblast homogenates. The results of this assay are shown in Fig. 3.9 The other experiment carried out to characterise the assay was performed in order to determine the inhibitory effect of tetrahydrouridine on the reaction. Figure 3.10 shows the inhibitory effect of increasing concentrations of THU on the reaction. THU was used in subsequent araC deaminase

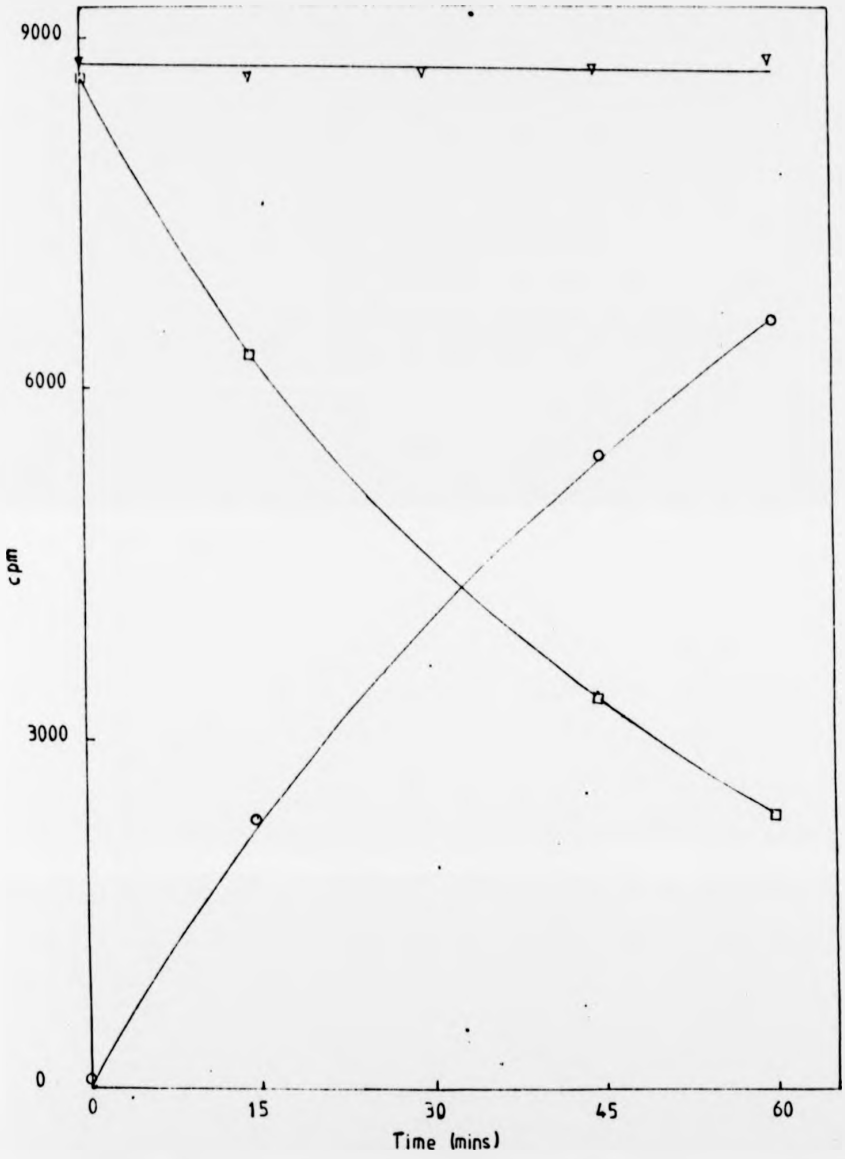



Fig 3.7 Characterisation of the araC deaminase assay (I). The Tetrahymena cell homogenate was incubated with $^{3}$H-araC for 0,15,30,45 and 60 minutes and araC deaminase activity assayed on DOWex-50 ion exchange columns as described in 3.4.6. 200ul samples were taken from the buffer and ammonia washes for each incubation period and the radioactivity in the samples determined. The radioactivity in the buffer wash (o) represents the ara U formed, the radioactivity in the ammonia wash (□)represents the remaining araC and (▽) is the total radioactivity from the two washes at each time interval.

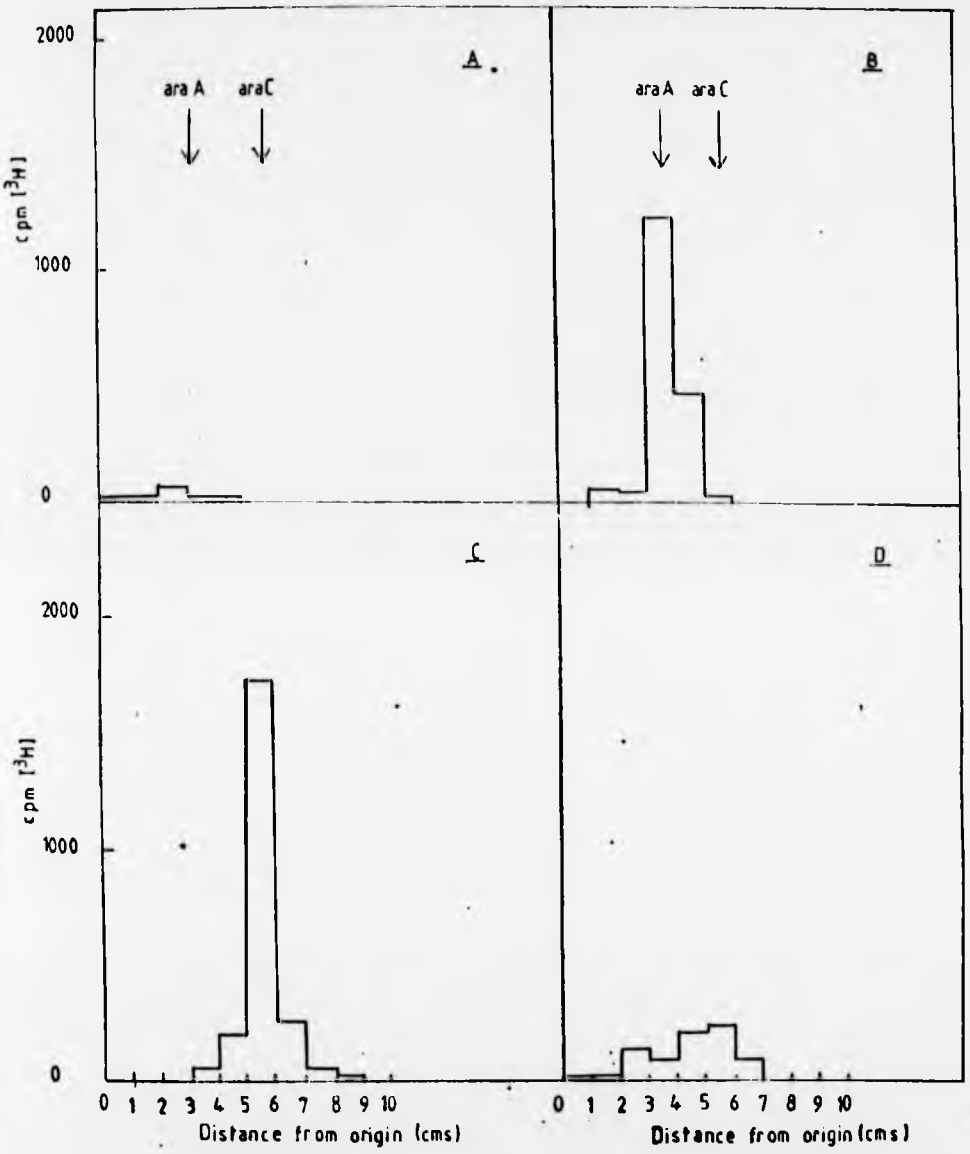



Fig 3.8 Characterisation of the araC deaminase assay (II). The assay products eluted from the DOWEX 50 columns were identified by descending paper chromatography as described in 3.4.6. The profiles shown are for samples taken from (A) the buffer wash after 0 minutes incubation, (B) the buffer wash after 60 minutes incubation, (C) the ammonia wash after 0 minutes incubation and (D) the ammonia wash after 60 minutes incubation.

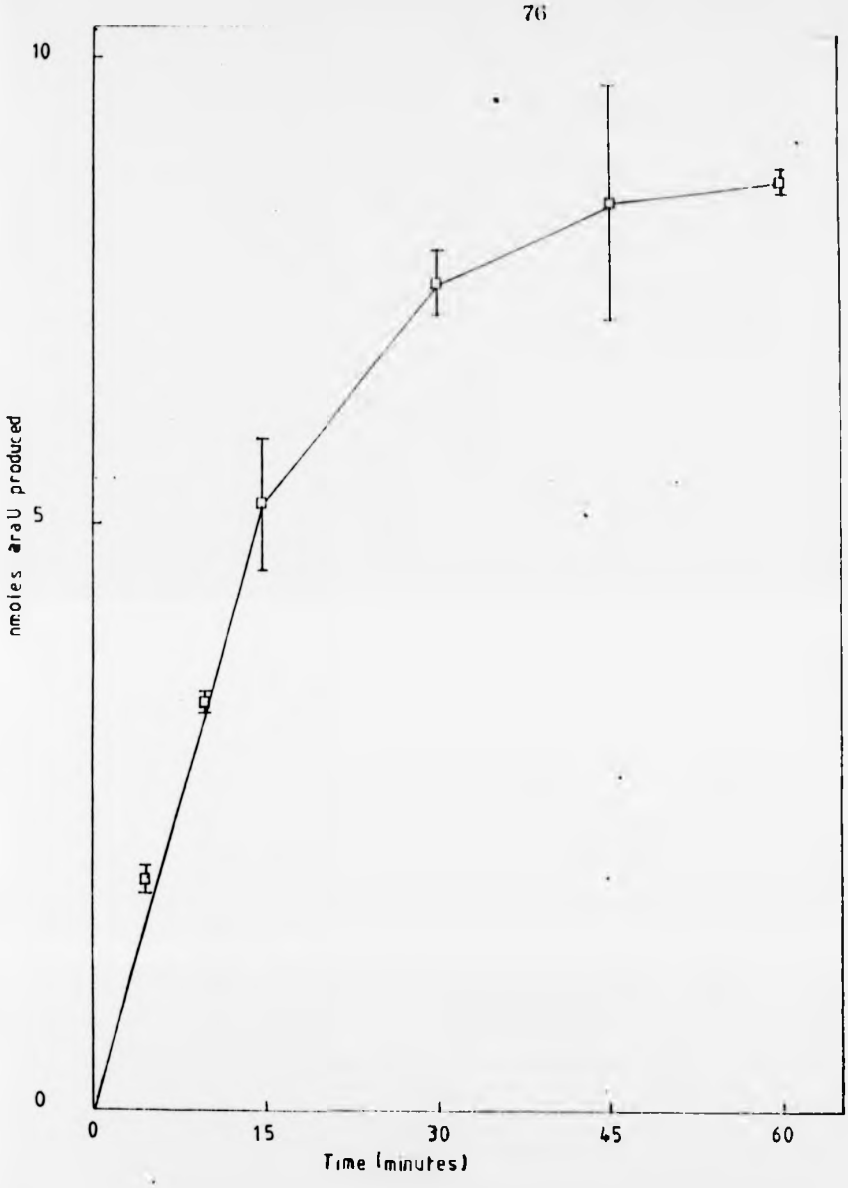



Fig 3.9 Time course for araC deaminase activity in T. pyriformis cell homogenate.

Errors represent the spread of the results about the mean, n=2

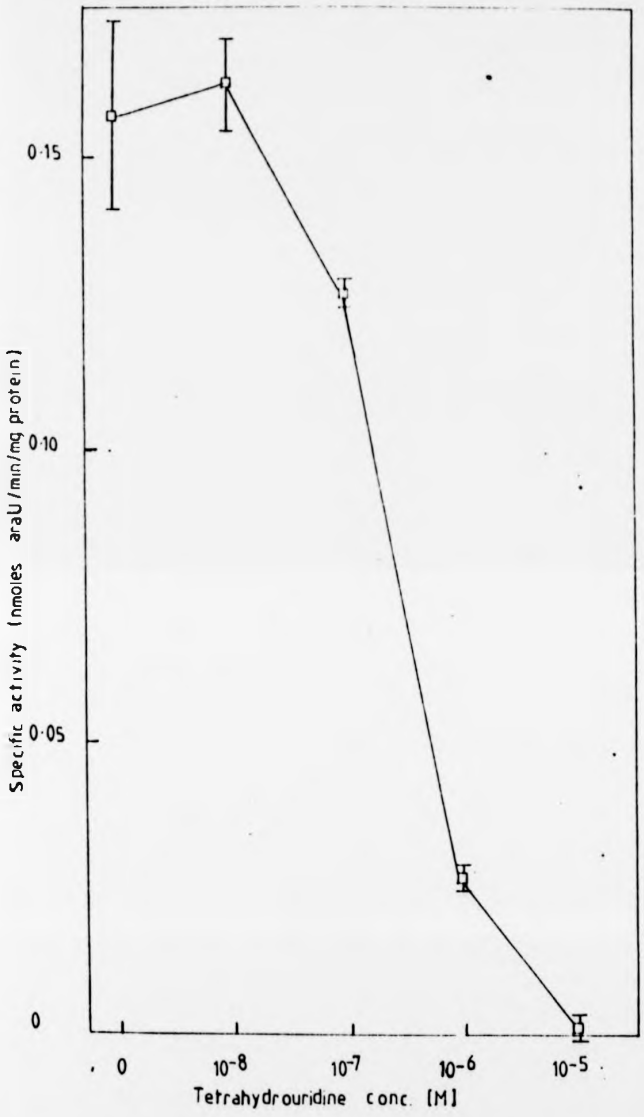



Fig 3.10 The inhibition of araC deaminase activity in T. pyriformis cell homogenate by tetrahydrouridine.
Errors represent the spread of the results about the mean, n=2

| Enzyme source | Deaminase activity nmoles/min/mg protein | Kinase activity nmoles/min/mg protein |
|---|---|---|
| T.pyriformis | 0.23 ± 0.01 | $0.29 \times 10^{-3}$ ± 0.01 |
| Namalwa cells | - | 0.52 ± 0.04 |
| CCRF/CEM cells | - | 0.22 ± 0.02 |

Fig 3.11 A comparison of the levels of araC deaminase and araC kinase activities in different cell types

Errors represent the spread of the results about the mean, $n=2$.

assays with the lymphoblast cell homogenates as an additional control. Inhibition of enzyme activity by THU was taken as a further confirmation of the presence of the araC deaminase enzyme.

### 3.5.5 Comparison of araC kinase and araC deaminase levels in different cell types

AraC deaminase and kinase assays were performed on cell homogenates of Nawalwa and CCRF-CEM cells and *T. pyriformis* as described in "Materials and Methods". The results from these assays show that only *T. pyriformis* possesses araC deaminase activity. THU inhibited araC deaminase in *T. pyriformis* but had no effect on the assay when lymphoblast cell homogenates were used. All the cell homogenates possess kinase activity, however the kinase activity in *T. pyriformis* is 1000X lower than that found in the lymphoblast cell homogenates. These results are consistent with the results obtained from the identification of araC metabolites using PEI thin layer chromatography.

## 3.6 DISCUSSION

The initial results indicate that araC is a potent inhibitor of DNA synthesis in Namalwa cells with 50% inhibition of thymidine incorporation into acid precipitable material being obtained with $5 \times 10^{-9}$ M araC. Tetrahydrouridine has little effect on thymidine incorporation and no effect on the inhibition of thymidine incorporation by $5 \times 10^{-9}$ M araC. If araC were being inactivated by a deaminase then the inhibitory effect of araC should be increased by the presence of a deaminase inhibitor such as tetrahydrouridine. This suggests (i) that THU inhibition of araC deaminase activity in Namalwa cells has negligible effect on araC inhibition or (ii) there is negligible araC deaminase activity in Namalwa cells. Subsequent

experiments were performed in an attempt to clarify this situation.

The metabolism of araC by *T. pyriformis* has been well characterised in this laboratory (M.A.N. Benhura, Ph.D. dissertation, University of Warwick, 1979). It is capable of rapidly deaminating araC and this deaminase activity is readily inhibited by THU. AraC has a poor inhibitory effect on *T. pyriformis* growth, this corresponds with a weak ability to convert araC to its phosphorylated derivatives. These findings have been confirmed in the control experiments carried out, both in the identification of the *T. pyriformis* metabolites of araC and in the characterisation of the araC deaminase assay using *T. pyriformis* cell homogenates.

Using these control experiments as references for comparison a study of the metabolism of araC in a T and B lymphoblastoid cell line was carried out. In studies on the metabolism of araC in the Namalwa, B cell, line it was found that araC was rapidly converted to the active triphosphate but there was no apparent conversion of araC to araU. The rapid phosphorylation of the drug to the active form correlates well with its high inhibitory effect on thymidine incorporation in Namalwa cells. The lack of araU among the araC metabolites further suggests that the absence of an araC deaminase as indicated by the negligible effect of THU on thymidine incorporation. It also appears as if the CCRF-CEM, T cell, line can rapidly convert araC to araCTP. There is no apparent deamination of araC to araU. Tetrahydrouridine has no effect on araC metabolism in either cell line as would be expected if there was no deaminase activity present. The direct assays for both the kinase and deaminase enzymes confirms the presence of the araC kinase enzyme in the lymphoblast cells and the absence of the deaminase enzyme. They also show the expected high deaminase levels and low kinase levels in *T. pyriformis*.

These results indicate that the levels of the enzymes in the activation and inactivation pathways are crucial for araC to have a useful inhibitory effect. The level of the deaminase enzyme can have an important effect on the inhibitory effect of araC but it may be possible to overcome this by the combination of the drug with a deaminase inhibitor such as THU. Ultimately however it appears as if the overriding factor is the level of the kinase enzyme. If this enzyme has only a low activity even inhibition of inactivating enzymes will only slightly affect the levels of the active phosphorylated form of the drug as is the case with *T. pyriformis*. However when kinase levels are high the drug can act as a potent inhibitor as illustrated by its effect on Namalwa cells.

These results have two further important consequences, one from a practical point of view with regards to the clinical use of araC and the other with regards to experimental studies on araC. These results suggest that araC may have a high therapeutic value as an anti-lymphocytic leukaemia and anti-lymphoma drug as well as being a potent drug in the treatment of myeloid leukaemias. Also the high kinase levels and low deaminase levels in the Namalwa cell lines and the potent effect of araC on this cell line make it a useful model system for the study of the mechanism of action of araC and its effect on eukaryotic DNA synthesis.

# CHAPTER 4

## THE MECHANISM OF ACTION OF CYTOSINE ARABINOSIDE

### 4.1 INTRODUCTION

Cytosine arabinoside is a known inhibitor of DNA synthesis (69,100). It has been shown to inhibit DNA synthesis in a number of eukaryotic cell systems such as mouse L cells (99,101), hamster embryo cells (102), PHA stimulated human lymphocytes (103) and rat liver (104). It has also been shown to be an inhibitor of *E. coli* DNA synthesis (104, 105) acting as an inhibitor of DNA polymerase III. AraC also inhibits RNA polymerase (106), reverse transcriptase of RNA viruses (96, 107), and glycolipid and glycoprotein synthesis (108), but its major mode of action has now been confirmed as an inhibitor of DNA synthesis.

Directly related to this observed effect on DNA synthesis is the observation that araC exerts its effect specifically during the S phase of the cell cycle (99, 109). The maximum sensitivity of synchronised mouse lymphoma cells to cytosine arabinoside occurs in early S phase (110). This knowledge has been used in *in vitro* studies on drug scheduling using drug regimens composed of araC in combination with other cell cycle specific drugs such as hydroxyurea and methotrexate (110, 111, 112, 113, 114). It has been found that improved therapeutic effects can be obtained when the scheduling of the drugs is directly related to cell cycle kinetics so that the drugs are administered during that part of the cell cycle where they have a maximum effect. This type of study has been extended to the *in vivo* situation in leukaemic mice by optimising the time of drug administration to suit the natural circadian activity of the mice (115).

The molecular mechanism by which araC inhibits DNA synthesis has not yet been fully resolved. One of the earliest proposed hypotheses was that araC inhibited the reduction of CDP to dCDP by the enzyme ribonucleotide reductase, thus blocking the production of dCTP and preventing DNA synthesis (116). However this hypothesis has now been discounted (99, 117). Another proposed hypothesis was that DNA synthesis was inhibited by the incorporation of araCTP into the 3'-OH termini of newly synthesised DNA strands causing premature termination of DNA replication. However it has been shown that araC, though incorporated into replicating DNA, was incorporated into internucleotide linkages. It was therefore not blocking the further addition of nucleotides to the growing DNA strand (101) and therefore could not be acting as an inhibitor by inducing premature termination.

The favoured, and most likely, hypothesis is that araCTP is an inhibitor of the eukaryotic DNA polymerase (96, 118, 119). Studies on cell-free systems and the isolated DNA polymerase enzymes have shown that the drug is a competitive inhibitor of DNA polymerase α with respect to dCTP and non-competitive with respect to dATP, dGTP and dTTP. DNA polymerase β is also slightly inhibited by araCTP, but is much less sensitive to the inhibitor than DNA polymerase α (23, 47, 120, 121, 122). This is in agreement with the fact that araC inhibits normal DNA replication, which is carried out by DNA polymerase α, rather than DNA repair synthesis, which is carried out by DNA polymerase β.

A similar pattern of inhibition is observed with adenosine arabinoside, the only difference being that DNA polymerase α is not so sensitive to araA inhibition as it is to araC (123, 124). AraA is a competitive inhibitor of DNA polymerase α with respect to dATP but non-competitive with respect to dCTP, dGTP and dTTP. One explanation for this is the concept of plural binding sites for the triphosphates

on the enzyme within the catalytic region so that the binding of a triphosphate at one site does not affect the binding of a triphosphate at another site. An alternative explanation is that the inhibitors may inhibit the action of the enzyme by binding at a site removed from the catalytic region and inhibiting the enzyme in an, as yet, unknown way (124).

If araCTP is an inhibitor of the replicative enzyme, DNA polymerase α, then it should inhibit the further elongation of newly initiated DNA chains. Several studies have been carried out using either whole cells or isolated nuclei systems in order to analyse the DNA produced under normal replicating conditions and in the presence of inhibitors such as cytosine arabinoside. Sedimentation analyses using such techniques as alkaline sucrose density gradient centrifugation and caesium chloride buoyant density centrifugation have been carried out so as to determine the size-class of DNA produced under different conditions. DNA is synthesised in short discrete lengths and ligated to give fully formed DNA as described in Chapter 2. Inhibition of DNA synthesis at different points in the replication process, i.e. initiation, elongation, termination and ligation, each lead to the accumulation of specific size-classes of DNA which can be identified using sedimentation analysis. Several workers have shown that the incubation of cell cultures or isolated nuclei with araC leads to the accumulation of low molecular weight DNA intermediates as would be expected if araC was inhibiting chain elongation (125, 126, 127).

Contrary to this expected behaviour of the DNA replication process in the presence of araC, Fridland has suggested that both araCTP and araATP inhibit the initiation process of DNA replication (128, 129). He has demonstrated that the incubation of CCRF-CEM lymphoblasts with

low concentrations of araC, 0.03 μM-0.05 μM, causes inhibition of the initiation process. This is shown by the accumulation of high molecular weight DNA intermediates in the presence of araC with a concommitant decrease in the amount of low molecular weight intermediates. It is suggested that araC may inhibit an enzyme involved in replicon initiation.

Another effect of araC on initiation has been proposed by Woodcock (130). He suggests that though araC is a potent inhibitor of DNA synthesis this is not enough to account for the cytotoxicity and cell death caused by araC treatment. This is supported by the fact that DNA synthesis *in vitro* can recover from araC inhibition and cells can regain the ability to synthesise new DNA chains after the removal of araC. It is proposed that cytotoxicity is a complex secondary phenomenon of the araC inhibition of DNA synthesis. Cytosine arabinoside causes damage to the DNA and severe chromosomal aberrations. The proposed mechanism by which this is achieved is that after receiving a pulse of araC, cells that have had their DNA replicative machinery inhibited, can re-initiate DNA synthesis in DNA segments that have already been replicated earlier in the S phase. This would mean that newly synthesised daughter duplexes would have two accompanying short DNA duplexes associated with them resulting in abnormal sections of replicating DNA which could in turn lead to DNA damage and chromosomal aberrations. This double replication of DNA segments following araC induced inhibition of DNA synthesis is not an effect of araC itself but a result of inhibiting DNA synthesis in the middle of replicon replication and therefore could be produced by other inhibitors of DNA synthesis. Similar effects have been observed with hydroxyurea.

The aim ofthe work in this chapter is to investigate the point in the DNA replication process where araC exerts its inhibitory effect,

i.e. initiation or elongation. The technique of alkaline surcrose density gradient centrifugation is used to identify the intermediates of DNA replication and the effect of araC on their synthesis.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Materials

Namalwa cells were cultured as described in 3.4.2. Sarkosyl NL35 was obtained from Ciba Geigy. Whatman 2.5 cm GF/C glass microfibre filters were obtained from Whatman Limited 2-$^{14}$C thymidine, [$^{3}$H-methyl], thymidine and 5-$^{3}$H cytosine-β-D-arabinoside were obtained from the Radiochemical Centre, Amersham. Cytosine arabinoside was obtained from the Sigma Chemical Company.

### 4.2.2 Cell incubation and labelling procedures

All incubations were carried out in Hepes buffered RPMI-1640 medium using 20 ml cultures of Namalwa cells, $10^{6}$ cells/ml. The 20 ml cultures were preincubated with 0.25 μCi, 2-$^{14}$C thymidine, sp. act. 21 Ci/mmol, for 24 hours. The cells were then resuspended in fresh medium and then incubated with 20 μCi [$^{3}$H-methyl] thymidine, sp. act. 25 Ci/mmol, under the following conditions:-

A(1) 4 hour incubation with $^{3}$H-thymidine.

(2) 4 hour incubation with $^{3}$H-thymidine in the presence of $5 \times 10^{-9}$ M araC.

B(1) 15, 30 and 60 minute incubations with $^{3}$H-thymidine.

(2) 15, 30 and 60 minute incubations with $^{3}$H-thymidine in the presence of $5 \times 10^{-9}$ M araC.

The following incubation procedure was also carried out in order to look at the possible incorporation of araC into intracellular DNA. 3 x 20 ml cultures of Namalwa cells, $10^{6}$ cells/ml, were incubated for 4 hours with 0.25 μCi 2-$^{14}$C thymidine. One culture was simultaneously

incubated with 20 μCi $^{3}$H-araC, sp. act. 26 Ci/mmol and $10^{-4}$ M cold araC for 4 hours. The other two cultures were incubated with 20 μCi $^{3}$H-araC and $10^{-4}$ M cold araC for the final 60 and 30 minutes of the 4 hour incubation.

### 4.2.3 Sedimentation analysis on alkaline sucrose gradients

After incubation the cells were harvested by centrifugation at 100 g, 5 minutes, 18-20°C and then washed with cold PBS and resuspended in 3 mls of 0.9% NaCl, 0.01M KCN, 0.008 M EDTA, 0.05 M potassium phosphate buffer pH 7.2. To this was added 500 μl Sarkosyl NL35 (35% Sarkosyl) to give a final concentration of 5% Sarkosyl. This was layered on to 5-20% alkaline sucrose gradients (sucrose in 10 mM EDTA, 0.2 M NaOH, 0.2 M NaCl) prepared on a 6 ml cushion of 60% sucrose and left overnight for the cells to lyse and the gradients to equilibrate. The gradients were centrifuged in an MSE 3 x 65 ml aluminium swing out rotor on an MSE Prepspin 65 ultracentrifuge at 22,000 r.p.m. (90,000 g) for 18 hours at 20°C. At the end of the 18 hour spin the centrifuge was allowed to stop with the brake off so as not to disturb the gradients. Fractions, volume 2 mls, were collected from the bottom of the gradient using a fine needle attached to an LKB 2120 varioperpex peristaltic pump and an LKB 2112 redirac fraction collector. 100 μl calf thymus DNA, 1 mg/ml in 5 mM NaOH, and 2 mls cold 10% TCA were added to the fractions. The fractions were left to stand overnight in the cold, 4°C. The acid-insoluble precipitates were collected by filtration on to Whatman GF/C glass microfibre filters and washed twice with 5 mls ethanol and dried in a warm oven. Scintillation fluid, 8 mls of Toluene, PPO 5 g/l, POPOP 0.2 g/l, was added to the discs and radioactivity determined for both $^{3}$H and $^{14}$C (see Appendix I). Sedimentation coefficient values were calculated by the method of McEwen (see Appendix II). All gradient profiles shown are

representative profiles obtained from a number of similar experiments.

## 4.3 RESULTS

### 4.3.1 The effect of araC on DNA synthesis in Namalwa cells

Namalwa cells, prelabelled with $^{14}$C-thymidine for a period of 24 hours, were incubated for 4 hours with $^{3}$H-thymidine in the presence of $5 \times 10^{-9}$ M araC and in the absence of araC. This concentration of araC is that which gave 50% inhibition of thymidine incorporation into acid precipitable material (see 3.5.1). The DNA produced under these conditions was analysed on alkaline sucrose gradients, the profiles obtained from these gradients are shown in Fig. 4.1. From these results it can be seen that $^{14}$C-thymidine has been incorporated into 25 S DNA. After a 4 hour incubation the $^{3}$H-thymidine was also found in 25 S DNA though the size of the 25 S peak was decreased by the presence of araC. No smaller size-classes of DNA were detected under these conditions.

In order to analyse these results quantitatively it was assumed that the area under the peaks (see Fig. 4.1) was proportional to the amount of labelled thymidine incorporated into a particular size-class of DNA and that the amount incorporated was proportional to the amount of DNA in that size-class. It was also observed that the area under the 25 S $^{14}$C peak was approximately the same in all of the gradients obtained from a particular set of incubation conditions. To refine the data on $^{3}$H-thymidine incorporation the areas under the control peaks of $^{14}$C-thymidine incorporation were all normalised by introducing a normalisation factor. Using this normalisation factor, the areas under the $^{3}$H peaks were calculated and corrected where necessary. The ratios of the areas under the $^{3}$H peaks for the different incubation conditions,

in this case with and without araC, should give an indication of the extent of the inhibition by araC. The positions of the peaks should indicate the site of inhibition by indicating which size-classes of DNA intermediates accumulated under a given set of conditions. The calculations for the gradients shown in Fig. 4.1 are given below. Similar calculations were used in the following sections to provide quantitative comparisons of the results obtained from sedimentation analyses from a number of different experiments.

Area under the 25 S $^{14}C$ peak obtained in the absence of araC = 10.8 $cm^2$ (1)

Area under the 25 S $^{14}C$ peak obtained in the presence of araC = 9.4 $cm^2$ (2)

The normalisation factor is obtained by dividing (1) by (2) = 1.14

Area under the 25 S $^{3}H$ peak obtained in the absence of araC = 6.25 $cm^2$ (3)

Area under the 25 S $^{3}H$ peak obtained in the presence of araC = 2.25 $cm^2$ (4)

Corrected value for (4) = 2.25 x 1.14 = 2.6 $cm^2$ (5)

The degree of inhibition obtained with 5 x $10^{-9}$ M araC can be obtained from the values of (3) and (5)

The percentage inhibition obtained $= 100 - (\frac{2.6}{6.25} \times 100)$

$= \underline{58\%}$

This value is comparable with that value obtained for the inhibition of $^{3}H$-thymidine incorporation into acid precipitable material by 5 x $10^{-9}$ M araC (see Fig. 5.3).

Occasionally very high counts for both $^{3}H$-thymidine and $^{14}C$-thymidine

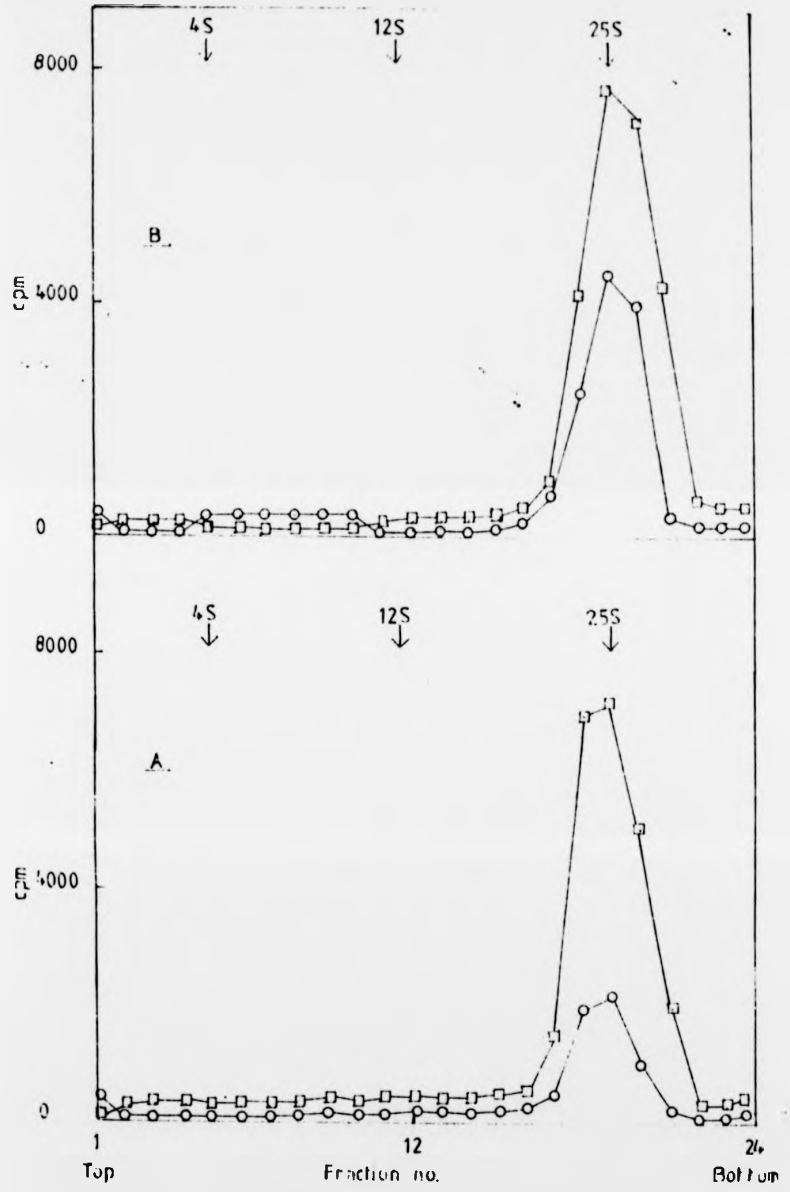



Fig 4.1 Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour pre-incubation with $^{14}$C-thymidine (□) followed by a 4 hour pulse with $^{3}$H-thymidine (o), A, in the presence of $5 \times 10^{-5}$ M cytosine arabinoside and B, without cytosine arabinoside. Gradient analysis was carried out as described in 4.2.3, sedimentation is from left to right.

were found in the 60% sucrose cushion. This probably was due to the pelleting of unlysed cells or fully formed high molecular weight DNA. The centrifugation conditions used were designed to look at the synthesis of low molecular weight DNA pieces.

### 4.3.2 The effect of araC on the synthesis of primary DNA pieces

Namalwa cells, prelabelled over a period of 24 hours with $^{14}$C-thymidine, were pulsed with $^{3}$H-thymidine for 15, 30 and 60 minutes and the DNA produced analysed on alkaline sucrose gradients (see Fig. 4.2). All the $^{14}$C-thymidine was found in the 25 S peak. After a 15 minute pulse, $^{3}$H-thymidine was found in 4 S DNA and 25 S DNA. 40% of the total $^{3}$H-thymidine incorporated into DNA was found in the 4 S peak and 60% in the 25 S peak. After a 30 minute pulse $^{3}$H-thymidine was found in 4 S, 12 S and 25 S DNA. The percentage of the total $^{3}$H-thymidine incorporated in each peak was 13%, 8% and 79% respectively. After a 60 minute pulse the $^{3}$H-thymidine was found predominantly in the 25 S peak, 92.5% of the total $^{3}$H-thymidine incorporated was found in this peak, 2.5% was found in the 4 S peak and 5% in the 12 S peak. The 4 S peak represents the primary, Okazaki, pieces of DNA formed as a result of the initiation of the synthesis of new replication units. The 12 S and 25 S peaks probably represent the DNA produced in the secondary elongation process arising from the arrangement of replication units either singly or in clusters (see Chapter 2).

This experiment was repeated but this time the $^{3}$H-thymidine pulses were carried out in the presence of $5 \times 10^{-9}$ M araC. The gradient profiles obtained are shown in Fig. 4.3. After a 15 minute pulse $^{3}$H-thymidine is only found in the 4S peak. The amount of $^{3}$H-thymidine in this peak is only 15% of the total $^{3}$H-thymidine

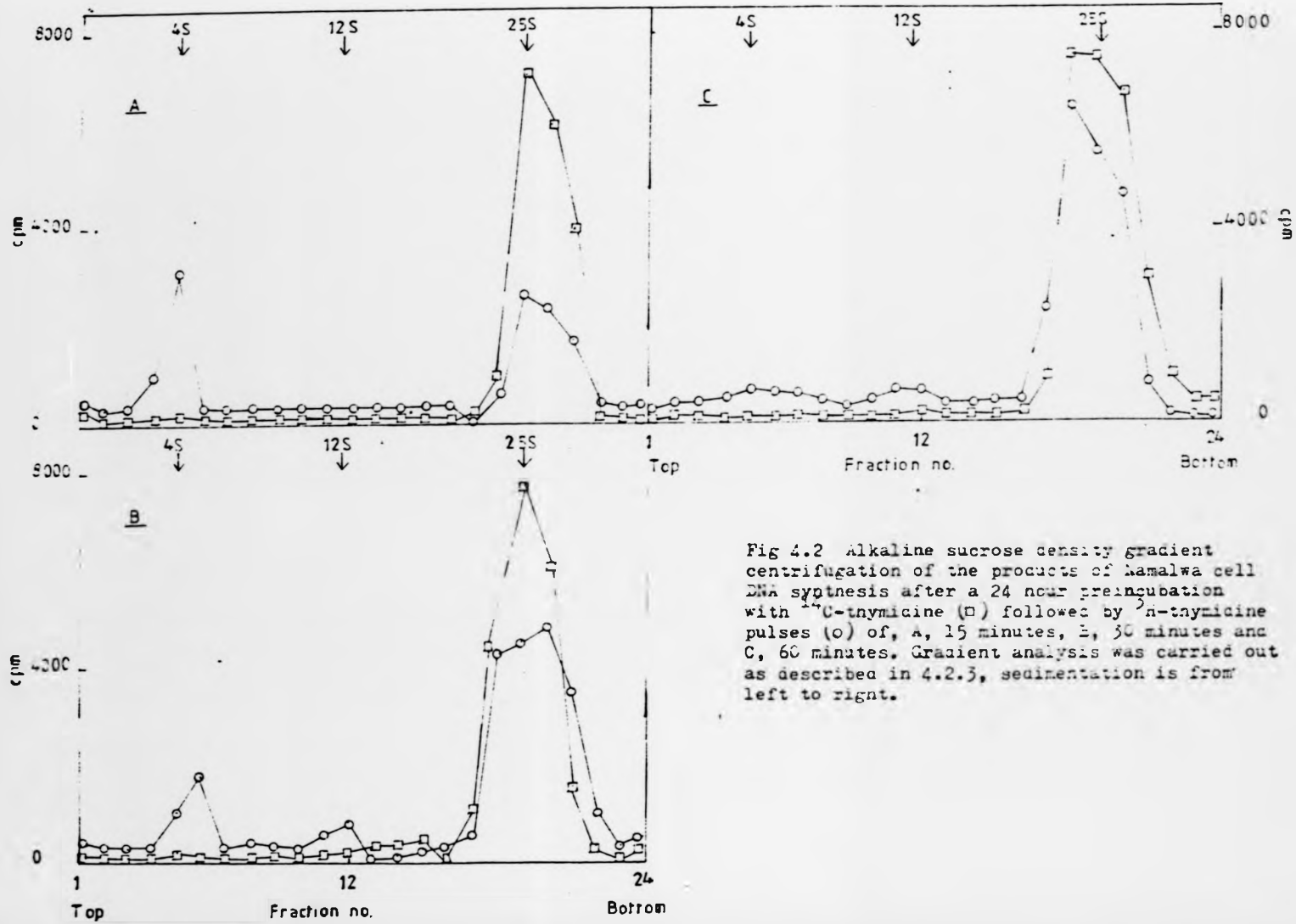



Fig 4.2 Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour preincubation with $^{14}$C-thymidine (□) followed by $^{3}$H-thymidine pulses (o) of, A, 15 minutes, B, 30 minutes and C, 60 minutes. Gradient analysis was carried out as described in 4.2.3, sedimentation is from left to right.

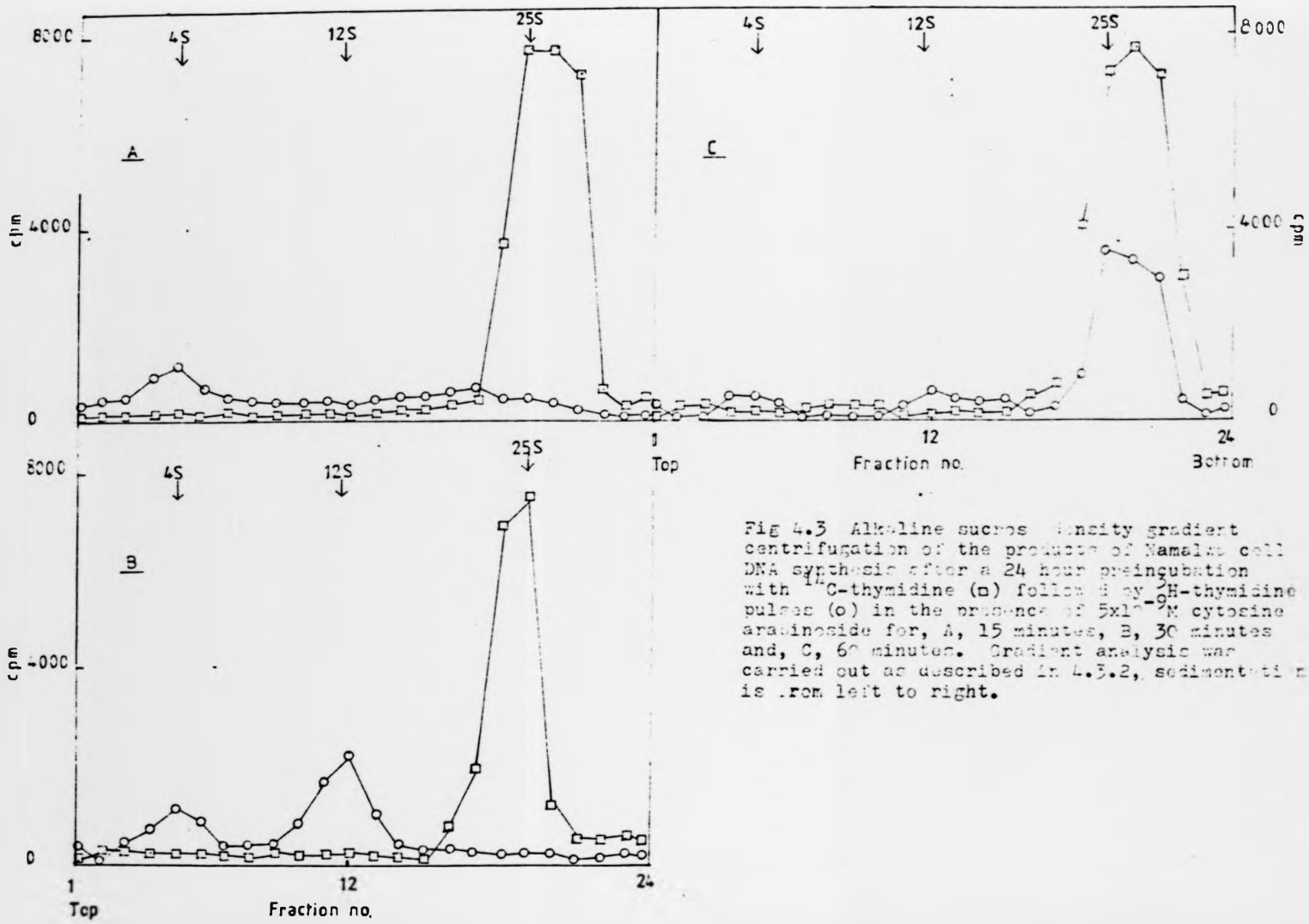



Fig 4.3 Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour preincubation with $^{14}$C-thymidine (□) followed by $^{3}$H-thymidine pulses (○) in the presence of $5 \times 10^{-6}$M cytosine arabinoside for, A, 15 minutes, B, 30 minutes and, C, 60 minutes. Gradient analysis was carried out as described in 4.3.2, sedimentation is from left to right.

incorporated into DNA after a 15 minute pulse without araC. After a 30 minute pulse the $^{3}$H-thymidine is found in the 4 S and 12 S peak, 75% of the total in the 12 S peak and 25% in the 4 S peak. The total thymidine incorporated is 60% of that found after a 30 minute pulse in the absence of araC. After a 60 minute pulse in the presence of araC the profile obtained is similar to that obtained after a 4 hour incubation with $^{3}$H-thymidine in the presence of araC, i.e. the majority of the $^{3}$H-thymidine, 82%, is found in the 25 S peak. The rest is evenly distributed throughout the 4 S and 12 S peaks. The heights of these two peaks are so small as to be almost negligible. The total $^{3}$H-thymidine incorporated after the 60 minute pulse is 68% of that found in the absence of araC representing a 42% inhibition of DNA synthesis.

The formation of small pieces of DNA in the presence of araC indicates that the initiation of DNA synthesis has not been inhibited. The rate of elongation has decreased in the presence of araC but has not been totally inhibited. This is to be expected as the araC concentration used was very low and was expected to only give 50% inhibition of DNA synthesis.

### 4.3.3 The incorporation of araC into newly synthesised DNA

Namalwa cells were incubated with $^{14}$C-thymidine for 4 hours and with 20 μCi $^{3}$H-araC at a final concentration of $10^{-4}$ M araC for the last 60 and 30 minutes of the 4 hour incubation. One culture was incubated with $^{14}$C-thymidine and $^{3}$H-araC simultaneously for 4 hours. The DNA produced was analysed on alkaline sucrose gradients. The gradient profiles obtained are shown in Fig. 4.4.

$^{14}$C-thymidine was found in the 4 S, 12 S and 25 S peaks after a

4 hour incubation during which $^{3}$H-araC had been added for the last 30 minutes. Most of the $^{14}$C-thymidine, 74%, was found in the 25 S peak, 23% was found in the 12 S peak and 3% in the 4 S peak. The $^{3}$H-araC was found evenly distributed between the 4 S and 12 S peaks. After a similar 4 hour incubation, but with $^{3}$H-araC present for the last 60 minutes of the incubation, again most of the $^{14}$C-thymidine, 81%, was found in the 25 S peak, 14% was found in 12 S DNA and 5% in 4 S DNA, i.e. a similar distribution to that found after a 30 minute exposure to araC. The araC was evenly distributed between the 4 S and 12 S peaks. After a simultaneous 4 hour incubation with $^{14}$C-thymidine and $10^{-4}$ M $^{3}$H-araC the $^{14}$C-thymidine was only found in 4 S and 12 S DNA, about 70% in the 4 S and 30% in the 12 S DNA. The total amount of incorporated $^{14}$C-thymidine under these conditions was also only 17% of that under the other two sets of incubation conditions, i.e. there was an 83% inhibition of $^{14}$C-thymidine incorporation. Again the $^{3}$H-araC was found evenly distributed throughout the 4 S and 12 S peaks but the amount of $^{3}$H-araC incorporated after the 30 and 60 minute incubations was only 30% of that found in the 4 hour exposure to $^{3}$H-araC.

It therefore appears as if araC has been incorporated into low molecular weight DNA, i.e. 4 S and 12 S. This again implies that araC has not inhibited the initiation of the synthesis of primary pieces of DNA. However araC has inhibited the synthesis of 25 S DNA. It therefore appears as if araC has inhibited the further elongation or ligation of DNA between the 12 S and 25 S stages under these conditions of high araC concentration.

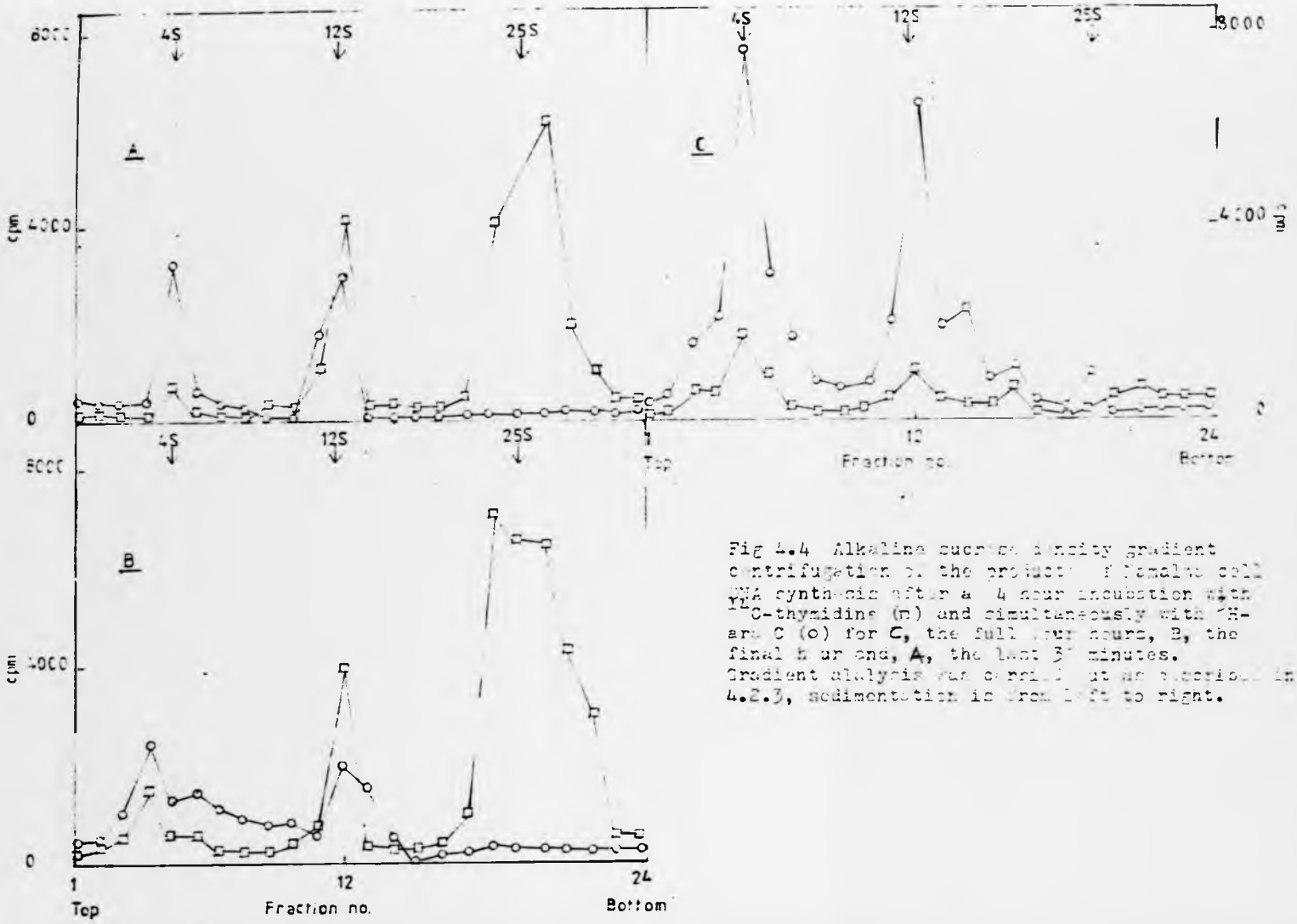



Fig 4.4 Alkaline sucrose density gradient centrifugation of the products of female cell DNA synthesis after a 4 hour incubation with $^{14}$C-thymidine (m) and simultaneously with $^{3}$H-and C (o) for C, the full four hours, B, the final hour and, A, the last 30 minutes. Gradient analysis was carried out as described in 4.2.3, sedimentation is from left to right.

## 4.4 DISCUSSION

The major mode of action of cytosine arabinoside is generally considered to be its inhibitory effect on the enzyme DNA polymerase α. This being the case one would expect araC to inhibit the elongation process of DNA synthesis as it is an inhibitor of the elongation enzyme. This is supported by the work of Wist *et al.*, Heligren *et al.* and Dijkwel and Wanka (125, 126, 127). However Fridland has proposed that the primary effect of araC, observable at low concentrations of the drug, is to inhibit the initiation of new replicons (128, 129). The results presented here are examined in the light of these two hypotheses.

The concentration of araC used in the following experiments ($5 \times 10^{-9}$ M) was chosen so that DNA synthesis was only partially inhibited. When lymphoblasts were incubated for 4 hours with $5 \times 10^{-9}$ M araC, all the $^{3}$H-labelled thymidine was found in the 25 S DNA region. This was the same result as in the control experiment without araC. However in the presence of the drug the 25 S peak was 58% smaller indicating a partial inhibition of DNA synthesis. When the cells were pulsed with $^{3}$H-thymidine for 15, 30 and 60 minutes in the presence of araC the rate of elongation was markedly inhibited compared to the control experiments without araC. However though the initiation process was slowed down under these conditions, it was by no means completely inhibited, whereas Fridland however reported the complete inhibition of initiation with $5 \times 10^{-8}$ M araC (129). The decreased rate of elongation from 4 S to 12 S to 25 S may represent an inhibition of the ligation process though this may be a secondary result of the slowing down of elongation by the inhibition of the polymerising activity of DNA polymerase α.

The incubation of Namalwa cells with $^{3}$H-araC and $^{14}$C-thymidine

resulted in the incorporation of araC into 4 S and 12 S DNA. This also coincides with the incorporation of $^{14}$C-thymidine into 4 S and 12 S DNA suggesting that they are both incorporated into the same growing DNA chains. The araC concentration used in this third set of experiments, $10^{-4}$ M, was chosen so as to give complete inhibition of DNA synthesis so that a more pronounced effect on the gradient profiles could be produced. The incorporation of $^{3}$H-araC into the 4 S and 12 S peaks again indicates that initiation has not been inhibited to any great extent. When the $^{3}$H-araC was incubated with the $^{14}$C-thymidine for 4 hours there was a complete inhibition of incorporation into 25 S DNA indicating a complete inhibition of the elongation process without an affect on the synthesis of 4 S and 12 S DNA showing that there was no significant inhibition of the initiation process even at this high concentration of araC. Presumably the araC is incorporated into DNA in place of deoxycytidine.

# CHAPTER 5

# THE MECHANISM OF ACTION OF APHIDICOLIN

## 5.1 INTRODUCTION

Aphidicolin is a tetracyclic, diterpenoid antibiotic produced by the mould *Cephalosporium aphidicola* Petch (131). It is a non-polar, water insoluble compound. Original studies on its biological activity showed that it was active against Herpes virus. Later work has shown that its anti-viral activity extends to other DNA viruses including SV40 and Vaccinia viruses (132, 133), but not Adenovirus (134). It also inhibits DNA synthesis in lymphocytes (135), HeLa cells (136), and other mammalian cells. It has no effect on *E. coli* DNA synthesis.

The principal mode of action of aphidicolin seems to be as an inhibitor of mammalian DNA polymerase α and certain DNA virus induced DNA polymerases involved in viral replication. It has no effect on DNA polymerase β or DNA polymerase γ. It therefore generally only affects normal, S phase, DNA replication and has little or no effect on DNA repair replication (132, 138). Inhibition studies have shown that it is a competitive inhibitor of DNA polymerase α with respect to dCTP, its inhibitory effect being reversed by the addition of excess dCTP (137, 138). It is a non-competitive inhibitor with respect to dGTP, dATP and dTTP.

However the picture is not as clear as depicted above. Seki *et al.*, have shown that aphidicolin can inhibit DNA repair synthesis in lymphocytes induced by UV irradiation or N-methyl-N'-nitro-N-nitroso guanidine (139). It is proposed, as a result of this evidence, that DNA polymerase α may play a role in DNA repair replication. A possible

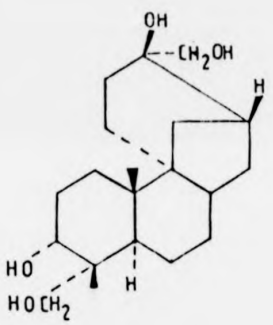



Fig 5.1 Aphidicolin

explanation for this discrepancy is that the DNA polymerase involved in repair may not be the same for all types of DNA damage or that there may be species differences in the repair enzymes involved. It is also suggested that the inhibition of DNA polymerase α may not be simple competitive inhibition with respect to dCTP but is a type of mixed-inhibition of competitive and non-competitive inhibition.

The competitive inhibition of DNA polymerase α by aphidicolin is similar to that observed with araCTP. Unfortunately this affords no clue as to the mechanism of action of aphidicolin as the chemical structure of aphidicolin is totally dissimilar to that of araCTP and NMR studies have shown that aphidicolin cannot form hydrogen bonds with guanine. It has been proposed by Oguro *et al.*, that there are separate binding sites for the four nucleotide triphosphates because (a) aphidicolin is only a competitive inhibitor with respect to dCTP and (b) studies using poly(dC).$(dG)_{12-18}$ and poly(dA).$(dT)_{12-18}$ as templates for DNA polymerase α show a reduced sensitivity to aphidicolin using dGTP and dTTP respectively as the labelled substrates for DNA polymerase α assays compared with activated DNA as a template-primer using dTTP as the labelled substrate. If there were not separate binding sites the degree of inhibition would be the same in all cases (138). Non-competitive inhibition with respect to dNTPs other than dCTP can be explained by the slower breakdown of the ternary enzyme-dNTP-aphidicolin complex. There are two possibilities for the aphidicolin binding site. The drug may bind competitively with dCTP at the dCTP binding site or it may bind at a site other than the catalytic site and compete with dCTP by an, as yet, unknown mechanism. The aphidicolin binding site may be in a hydrophobic region of the enzyme molecule as it is a non-polar compound.

Aphidicolin, because of its specificity for eukaryotic DNA

polymerase α, is a useful tool for studying eukaryotic DNA replication. In this chapter its effect on thymidine incorporation in Namalwa cells is investigated. This is used as a preliminary study leading to an investigation of its effect on DNA synthesis in Namalwa lymphoblastoid cells.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Materials

Aphidicolin was a gift from Dr G Coles, I.C.I. Pharmaceuticals, Alderly Edge. The source of all other materials used was the same as described in 4.2.1.

### 5.2.2 The effect of aphidicolin on thymidine incorporation in Namalwa cells

1 ml cultures of Namalwa cells, $10^6$ cells/ml, were incubated at 37°C for four hours with [$^3$H-methyl] thymidine, sp. act. 47 Ci/mmol, as described in 3.4.4. Aphidicolin, dissolved in DMSO, was added at final concentrations of 0, $10^{-9}$, $10^{-8}$, $5 \times 10^{-8}$, $10^{-7}$, $5 \times 10^{-7}$, $10^{-6}$ and $10^{-5}$ M, so that the final concentration of DMSO was not greater than 1%. The incorporation of radioactive thymidine into acid precipitable material was assayed as described in 3.4.4. Results are shown as the percentage of maximum thymidine incorporation into acid precipitable material.

### 5.2.3 Cell incubation and labelling procedures for sedimentation analysis

All incubations were carried out in Hepes buffered RPMI-1640 medium using 20 ml cultures of Namalwa cells, $10^6$ cells/ml. The 20 ml cultures were preincubated for 24 hours with 0.25 μCi $^{14}$C-thymidine, sp. act. 21 Ci/mmol. The cells were then resuspended in fresh medium

and incubated with 20 μCi [$^{3}$H-methyl] thymidine, sp. act. 25 Ci/mmol, under the following conditions:

(1) 4 hour incubation with $^{3}$H-thymidine.

(2) 4 hour incubation with $^{3}$H-thymidine in the presence of $10^{-7}$ M aphidicolin.

(3) 15, 30 and 60 minute incubations with $^{3}$H-thymidine in the presence of $10^{-7}$ M aphidicolin.

Sedimentation analysis on 5–20% alkaline sucrose gradients was carried out as described in 4.2.3.

## 5.3 RESULTS

### 5.3.1 The effect of aphidicolin on $^{3}$H-thymidine incorporation by Namalwa cells

The effect of increasing concentrations of aphidicolin on the incorporation of $^{3}$H-thymidine into acid precipitable material is shown in Fig. 5.2 Aphidicolin is not such a potent inhibitor of DNA synthesis in Namalwa cells as cytosine arabinoside but it is still effective at low concentrations. 50% inhibition of thymidine incorporation is obtained with $10^{-7}$ M aphidicolin. Inhibition was seen with concentrations as low as $10^{-8}$ M and 100% inhibition was attained with $10^{-5}$ M aphidicolin.

### 5.3.2 The effect of aphidicolin on DNA synthesis in Namalwa cells

Namalwa cells, prelabelled with $^{14}$C-thymidine, were incubated for 4 hours with $^{3}$H-thymidine in the presence of $10^{-7}$ M aphidicolin and in the absence of aphidicolin. The concentration of aphidicolin used is that which gave 50% inhibition of thymidine incorporation into acid precipitable material (see 5.3.1). The DNA produced under these

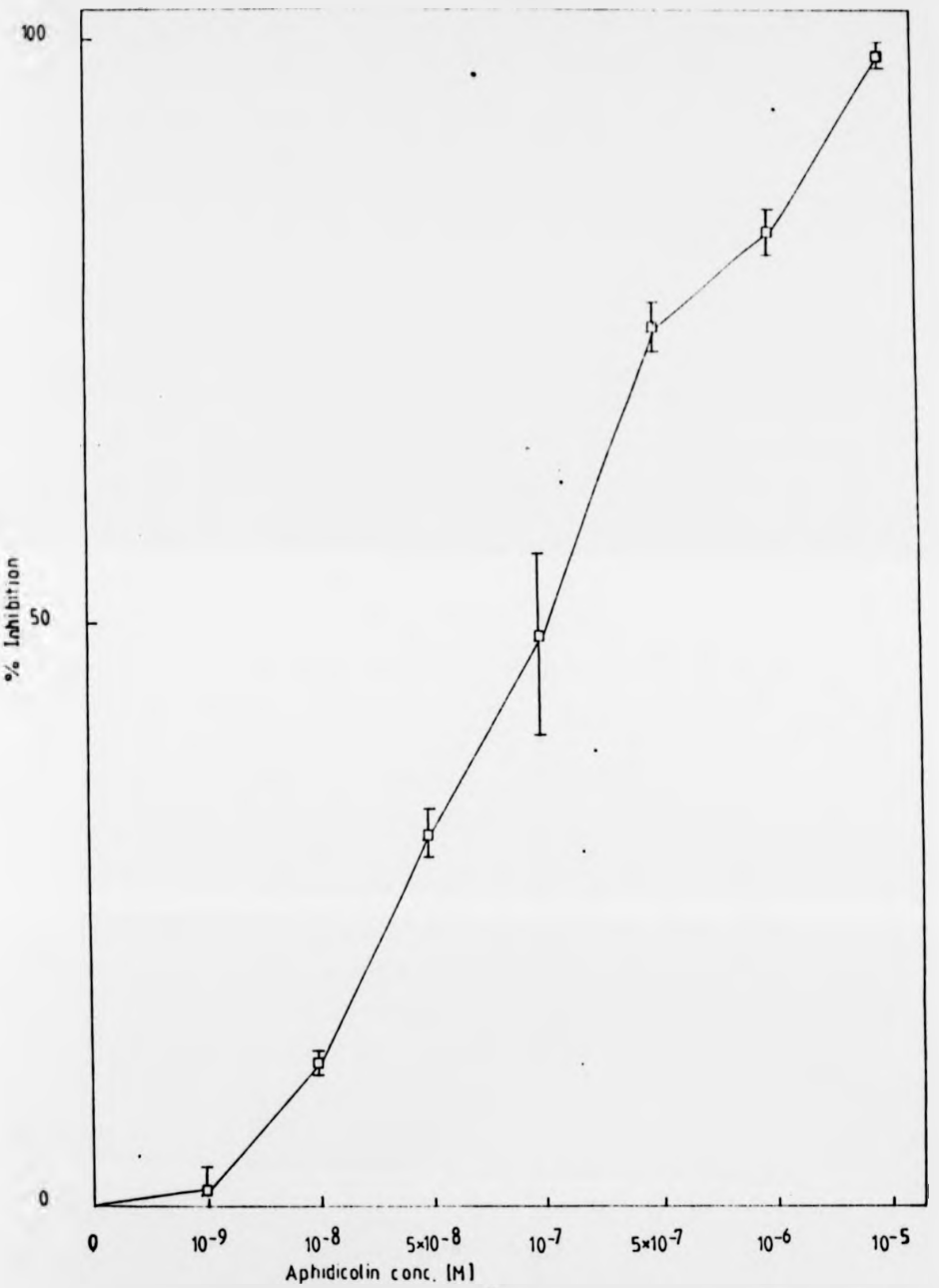



Fig 5.2 The inhibition of $^{3}$H-thymidine uptake in Namalwa cells by aphidicolin. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E., n=3.

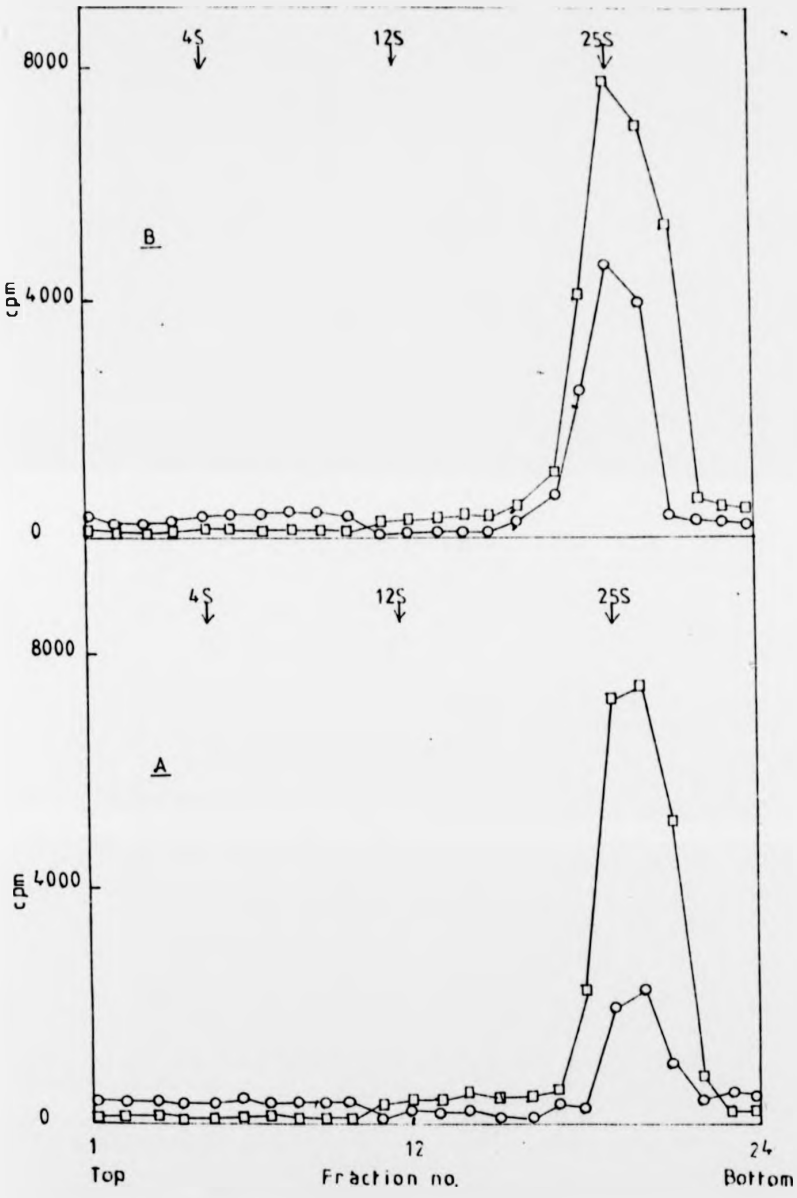



Fig 5.3 Alkaline sucrose density gradient centrifugation of the products of Namalva cell DNA synthesis after a 2½ hour preincubation with $^{14}$C-thymidine (□) followed by a 4 hour pulse with $^{3}$H-thymidine (○), A, in the presence of $10^{-7}$M aphidicolin and, B, without aphidicolin. Gradient conditions carried out as described in 4.2.3, sedimentation is from left to right.

conditions was analysed on alkaline sucrose gradients. The gradient profiles are shown in Fig. 5.3. All the $^{14}$C-thymidine was incorporated into 25 S DNA. After the 4 hour incubation all the $^{3}$H-thymidine was also found in 25 S DNA in experiments both with and without aphidicolin. The size of the $^{3}$H-thymidine 25 S peak formed in the presence of aphidicolin was 54% smaller than that produced in the control incubation without aphidicolin. This indicates 54% inhibition of DNA synthesis in Namalwa cells treated with $10^{-7}$ M aphidicolin. There was no thymidine incorporation into 4 S or 12 S DNA under these conditions.

### 5.3.3 The effect of aphidicolin on the synthesis of DNA primary pieces

Namalwa cells, prelabelled with $^{14}$C-thymidine, were pulsed with $^{3}$H-thymidine in the presence of $10^{-7}$ M aphidicolin for 15, 30 and 60 minutes and the DNA produced analysed on alkaline sucrose gradients (see Fig. 5.4). All the incorporated $^{14}$C-thymidine was found in 25 S DNA. After a 15 minute pulse a small amount of $^{3}$H-thymidine was found in the 4 S and 12 S DNA peaks, 45% of the total $^{3}$H-thymidine incorporated was found in the 4 S peak and 55% in the 12 S peak. The total amount of $^{3}$H-thymidine incorporated is 32% of that obtained under similar conditions with no drug present (see Fig. 4.2). After a 30 minute pulse the total amount of thymidine incorporated had increased, but radioactivity was still only found in 4 S and 12 S DNA regions. 60% was found in 4S DNA and 40% in 12 S DNA. The total amount of thymidine incorporated under these conditions was 46% of that found under similar conditions with no drug present. At the end of the 60 minute pulse the majority of the $^{3}$H-thymidine, 82%, was found in the 25 S peak and 18% in the 4 S peak. There was no incorporation of thymidine into 12 S DNA. The total amount of $^{3}$H-thymidine incorporated into DNA was 47% of that found in the control

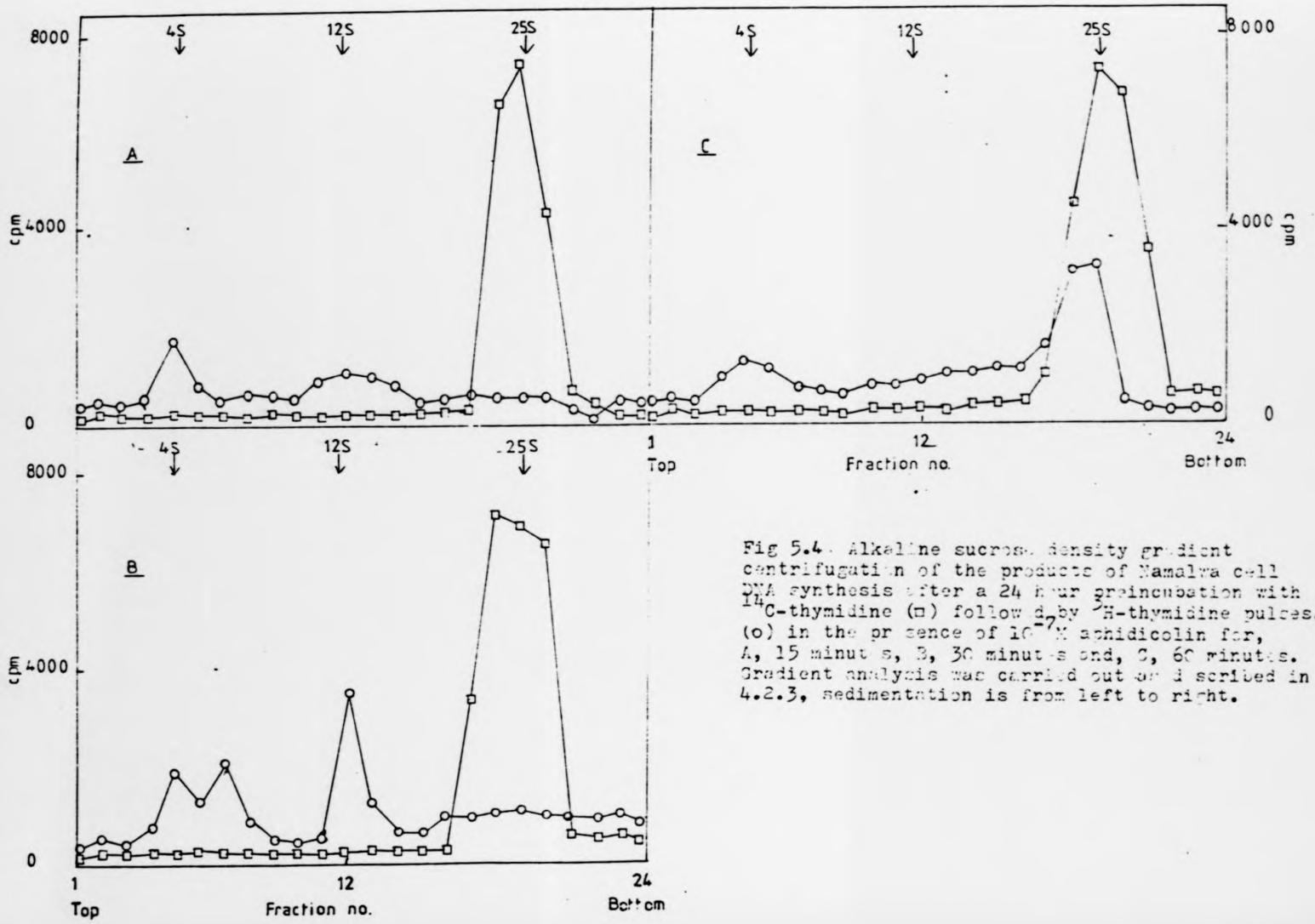



Fig 5.4. Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour preincubation with $^{14}$C-thymidine (□) followed by $^{3}$H-thymidine pulses. (o) in the presence of $10^{-7}$M aphidicolin for, A, 15 minutes, B, 30 minutes and, C, 60 minutes. Gradient analysis was carried out as described in 4.2.3, sedimentation is from left to right.

without any drug present. This represents a 53% inhibition of DNA synthesis with $10^{-7}$ M aphidicolin.

## 5.4 DISCUSSION

Aphidicolin is a known inhibitor of DNA polymerase α. This property of aphidicolin has been used as a tool to study DNA synthesis. Being an inhibitor DNA polymerase α it should only affect the elongation process of DNA synthesis. It therefore provides a useful comparison for the study of the inhibition of DNA synthesis with araC.

The result shown in Fig. 5.2 demonstrates the inhibition of DNA synthesis in Namalwa cells by aphidicolin as measured by the incorporation of radioactive thymidine into acid precipitable material. 50% inhibition was obtained with $10^{-7}$ M aphidicolin. This concentration of drug was chosen for the study of the effect of aphidicolin on the process of DNA synthesis. This degree of inhibition was the same as that given by the araC concentration used in the experiments described in 4.3.1 and 4.3.2 thus allowing direct comparisons to be made.

After a four hour incubation of lymphoblasts with $^{3}$H-thymidine in the presence of $10^{-7}$ M aphidicolin all the labelled thymidine was found in 25 S DNA (Fig. 5.3). A similar result was achieved by repeating the experiment without aphidicolin. However the presence of the drug decreased the size of the $^{3}$H-thymidine 25 S peak by 54%. This indicated an inhibition of DNA synthesis of the order of 50% as would be predicted from the thymidine incorporation study (5.3.1).

When the $^{14}$C-thymidine prelabelled cells were pulsed with $^{3}$H-thymidine for 15, 30 and 60 minutes in the presence of $10^{-7}$ M aphidicolin the rate of elongation was inhibited compared with the control experiments without drug (Fig. 4.2). There was a decrease in

the rate of initiation but this was probably the result of the decreased rate of elongation. The gradient profiles produced under these conditions (Fig. 5.4) were similar to those produced under similar conditions with $5 \times 10^{-9}$ M araC. This suggests that the mechanism of inhibition of the two drugs may be similar. As aphidicolin is an inhibitor of the elongation enzyme, DNA polymerase α, it is proposed that araC is also primarily an inhibitor of DNA chain elongation.

# CHAPTER 6

## CYCLOPHOSPHAMIDE AND DNA SYNTHESIS

### 6.1 THE MECHANISM OF ACTION OF CYCLOPHOSPHAMIDE

Cyclophosphamide is a widely used, potent, anti-cancer drug and is of the class of drugs known as the alkylating agents. It is a phosphoramide derivative of the nitrogen mustards, a group of potent and very toxic alkylating reagents. Nitrogen mustards alkylate by a two step process. The first stage is the rapid cyclisation of the molecule to give an ethyleneimmonium ion. The second step is slower and is an $S_N2$ alkylating step. This is the rate determining step in the alkylation mechanism. Phosphoramide mustards probably alkylate via the same mechanism as the nitrogen mustards (140). The postulated mechanism of alkylation of phosphoramide mustard is shown in Fig. 6.1.

Cyclophosphamide itself is inactive as an alkylating agent and has to be first metabolised to an active form (14, 141). The principal site of activation is the liver where cyclophosphamide is metabolised by oxidative microsomal enzymes. The major metabolites of cyclophosphamide are shown in Fig. 6.2. The first activation step is the oxidation of the phosphoramide ring to produce 4-hydroxycyclophosphamide. This compound can then be detoxified by further oxidation to the inactive 4-ketocyclophosphamide. Alternatively the 4-hydroxycyclophosphamide can tautomerise to the open chain aldehyde, aldophosphamide. This can also undergo further oxidation to give carboxyphosphamide. The ketocyclophosphamide and carboxyphosphamide are the major urinary metabolites excreted by the body and represent 15% and 50% respectively of the administered drug. Alternatively the aldophosphamide can be cleaved non-enzymatically via spontaneous

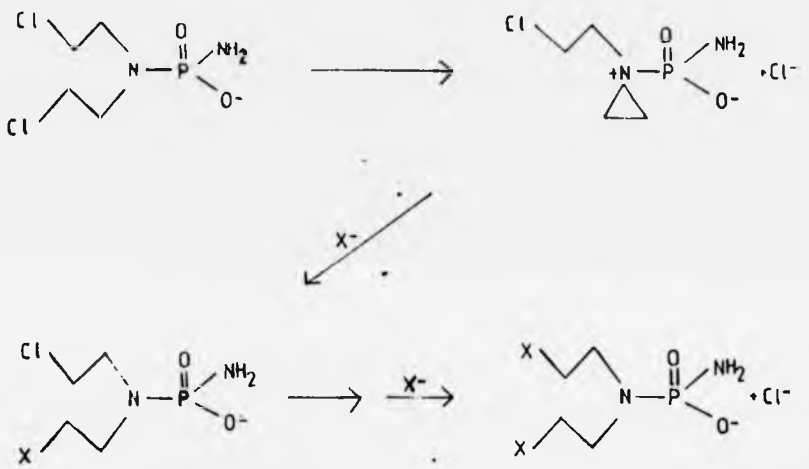



Fig 6.1 The mechanism of alkylation by phosphoramide mustard of compound, X, via postulated cyclic ethylenimmonium ion intermediate

β elimination of acrolein to give phosphoramide mustard, the postulated active form of the drug. The metabolism of cyclophosphamide can be altered by drugs affecting microsomal metabolism, e.g. phenobarbitone increases microsomal enzyme activity in rats giving accelerated production of the alkylating metabolites, whereas the antibiotic chroramphenicol inhibits microsomal enzymes and produces the reverse effect (14).

The alkylating activity of phosphoramide mustard cannot alone account for the potency of cyclophosphamide. It is thought that some of the metabolites of cyclophosphamide such as 4-hydroxycyclophosphamide, aldophosphamide and iminophosphamide may act as carriers of phosphoramide mustard which, on entering the target cell, can release phosphoramide mustard (141).

Phosphoramide mustard is a non-specific alkylating agent and the exact target of the drug is not known, however the favoured target is DNA (142). It is thought that phosphoramide mustard, being a bifunctional alkylating agent, can cross-link the two strands of the DNA double helix thus preventing DNA replication. Because of its non-specific action cyclophosphamide is not cell-cycle specific like the other inhibitors of DNA synthesis, araC and aphidicolin, that have already been examined. This lack of specificity makes it difficult to explain the observed tumour cell specificity of cyclophosphamide over normal cells. Two main theories have been proposed to account for this (141). One theory proposes that cyclophosphamide and its metabolites are bound by thiols, probably proteins, giving increased metabolic stability so that entry into target cells is facilitated. This thiol binding also enables the metabolites to be directed to specific sites in the cells and gives a delayed release of phosphoramide mustard into the target cell. The other hypothesis

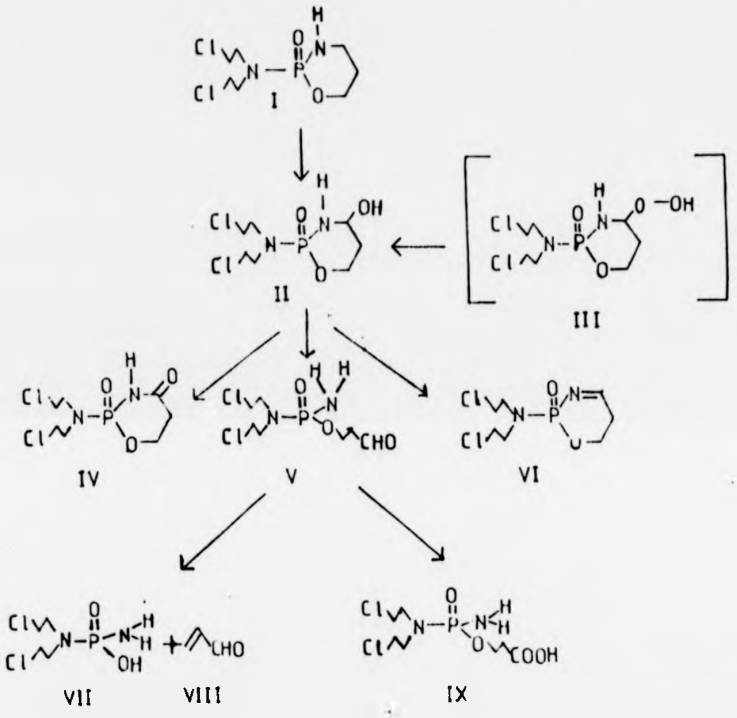



Fig 6.2 The metabolism of cyclophosphamide. Cyclophosphamide (I), 4, hydroxycyclophosphamide (II), 4, hydroperoxy-cyclophosphamide (III), 4, ketocyclophosphamide (IV), aldophosphamide (V), iminophosphamide (VI) phosphoramide mustard (VII), acrolein (VIII), carboxyphosphamide (IX)

proposes that cyclophosphamide and its metabolites are selectively detoxified by normal cells thus rendering them inactive. This detoxification mechanism is thought not to exist in tumour cells thus making them susceptible to the alkylating agents.

Several derivatives of cyclophosphamide have been synthesised in this laboratory including the non-physiological derivative hydroperoxy-cyclophosphamide (see Fig. 6.2). This spontaneously decomposes in water to the activated, hydroxy, form of the drug (143) thus providing an active form of the drug that can be used in *in vitro* studies. Extensive work has been carried out in this laboratory on the effect of cyclophosphamide and its derivatives on cyclic nucleotide metabolism, and RNA and protein synthesis. This chapter looks at the effect of cyclophosphamide and its derivatives on the DNA synthesis of the transformed lymphoblastoid cell line.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Materials

Cyclophosphamide was obtained from Boehringer Mannheim. Phosphoramide mustard (cyclohexylamine salt) was obtained from NIH, Bethesda, Maryland. Hydroperoxycyclophosphamide was synthesised by L.A. Fitton and G.J. Hunter, Department of Chemistry and Molecular Sciences, University of Warwick. All other materials were obtained as described in 4.2.1.

### 6.2.2 The effect of alkylating agents on thymidine incorporation in Namalwa cells

1 ml cultures of Namalwa cells, $10^6$ cells/ml, were incubated at 37°C for one hour with [$^3$H-methyl] thymidine, sp. act. 47 Ci/mmol. All the alkylating agents, dissolved in PBS, were added to give the

following final concentrations: 0, $10^{-5}$, $10^{-4}$, $5 \times 10^{-4}$, $10^{-3}$ and $5 \times 10^{-3}$ M. The incorporation of radioactive thymidine into acid precipitable material was assayed as described in 3.4.4. Results are shown as the percentage of maximum thymidine incorporation into acid precipitable material.

### 6.2.3 Cell incubation and labelling procedures for sedimentation analysis

All incubations were carried out in Hepes buffered RPMI 1640 medium using 20 ml cultures of Namalwa cells, $10^6$ cell/ml. The 20 ml cultures were preincubated for 24 hours with 0.25 µCi $^{14}$C-thymidine, sp. act. 21 Ci/mmol. The cells were then resuspended in fresh medium and cinbuated for one hour with 20 µCi [$^3$H-methyl] thymidine, sp. act. 25 Ci/mmol and (1) $10^{-3}$ M cyclophosphamide (2) $10^{-3}$ M phosphoramide mustard (3) $10^{-3}$ M hydroperoxycyclophosphamide and (4) without drug. Sedimentation analysis on 5-20% alkaline sucrose gradients was carried out as described in 4.2.3.

## 6.3 RESULTS

### 6.3.1 The effect of alkylating agents on $^3$H-thymidine incorporation by Namalwa cells

The effect of cyclophosphamide, phosphoramide mustard and hydroperoxycyclophosphamide on $^3$H-thymidine incorporation into acid preciptable material is shown in Fig. 6.3. The period of incubation for these assays was one hour, rather than four hours as described in 3.4.4, as phosphoramide mustard is rapidly degraded in aqueous solution. Cyclophosphamide has little effect on $^3$H-thymidine incorporation into acid precipitable material. Only 20% inhibition was obtained with $5 \times 10^{-3}$ M cyclophosphamide which was the maximum concentration used. Phosphoramide mustard has a greater effect than

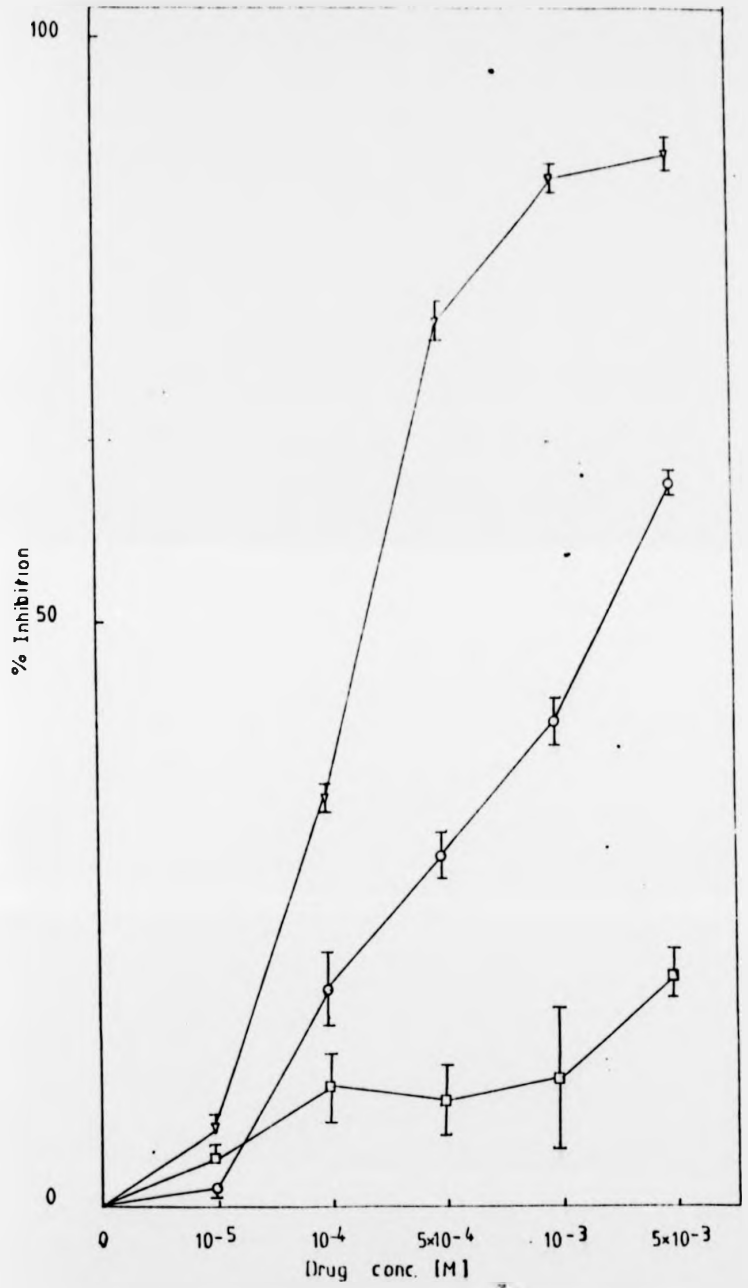



Fig 6.3 The inhibition of $^{3}$H-thymidine uptake in Namalwa cells by cyclophosphamide (□), phosphoramide mustard (○) and hydroperoxycyclophosphamide (▽). Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described 3.4.4. Error bars represent S.E., n=3

cyclophosphamide. As the concentration of phosphoramide mustard increases so inhibition increases starting at 18% inhibition with $10^{-4}$ M up to 60% inhibition with $5 \times 10^{-3}$ M. The greatest inhibitory effect was seen with hydroperoxycyclophosphamide. $10^{-4}$ M hydroperoxycyclophosphamide gave 35% inhibition and as the drug concentration increased so the degree of inhibition increased so that 90% inhibition was obtained with $5 \times 10^{-3}$ M of the compound.

### 6.3.2 The effect of alkylating agents on DNA synthesis in Namalwa cells

Four cultures of Namalwa cells, prelabelled with $^{14}$C-thymidine, were incubated for 1 hour with $^{3}$H-thymidine and (A) without drug, (B) with $10^{-3}$ M cyclophosphamide, (C) $10^{-3}$ M phosphoramide mustard and (D) $10^{-3}$ M hydroperoxycyclophosphamide. The DNA produced was analysed on alkaline sucrose gradients. The gradient profiles obtained are shown in Fig. 6.4a and 6.4b. $^{3}$H-thymidine was only incorporated into 25 S DNA when the Namalwa cells were incubated in the absence of the alkylating agents. When the cells were incubated with $10^{-3}$ M cyclophosphamide $^{3}$H-thymidine was incorporated into 4 S, 12 S and 25 S DNA. 69% of the label was incorporated into 25 S DNA. 15% into 12 S DNA and 16% into 4 S DNA.

When the cells were incubated with $10^{-3}$ M phosphoramide mustard $^{3}$H-thymidine was incorporated into 4 S and 25 S DNA. There was also $^{3}$H-thymidine in a 19 S DNA peak that had not been previously observed in any of the other gradient analyses. 64% of the $^{3}$H-thymidine was in 25 S DNA, 11% in 4 S DNA and 25% in 19 S DNA. When the cultures were incubated with $10^{-3}$ M hydroperoxycyclophosphamide all the incorporated $^{3}$H-thymidine was found in the 19-20 S DNA peak. The 25 S $^{14}$C-thymidine peak was also broader.

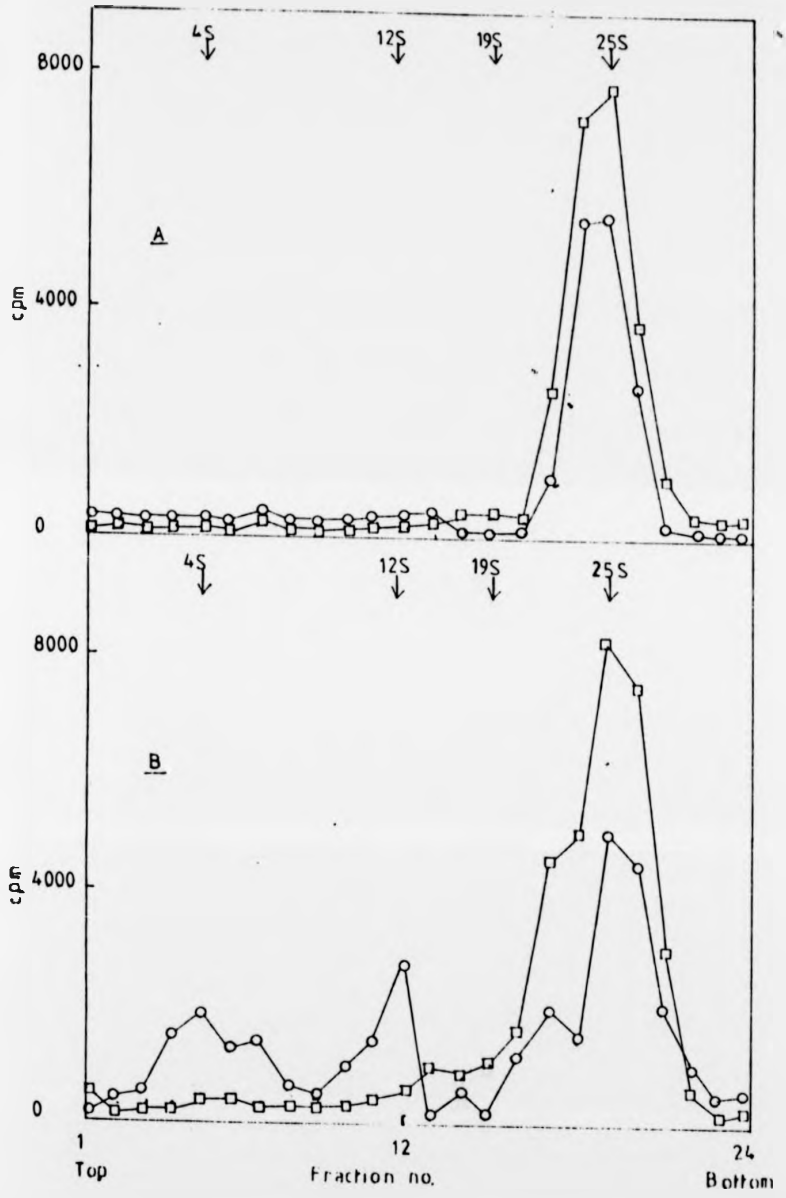



Fig 6.4a Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour preincubation with $^{14}$C-thymidine (□) followed by a 1 hour pulse of $^{3}$H-thymidine (○), A, without drugs and B, in the presence of $10^{-3}$M cyclophosphamide. Gradient analysis was carried out as described in 4.2.5, sedimentation is from left to right.

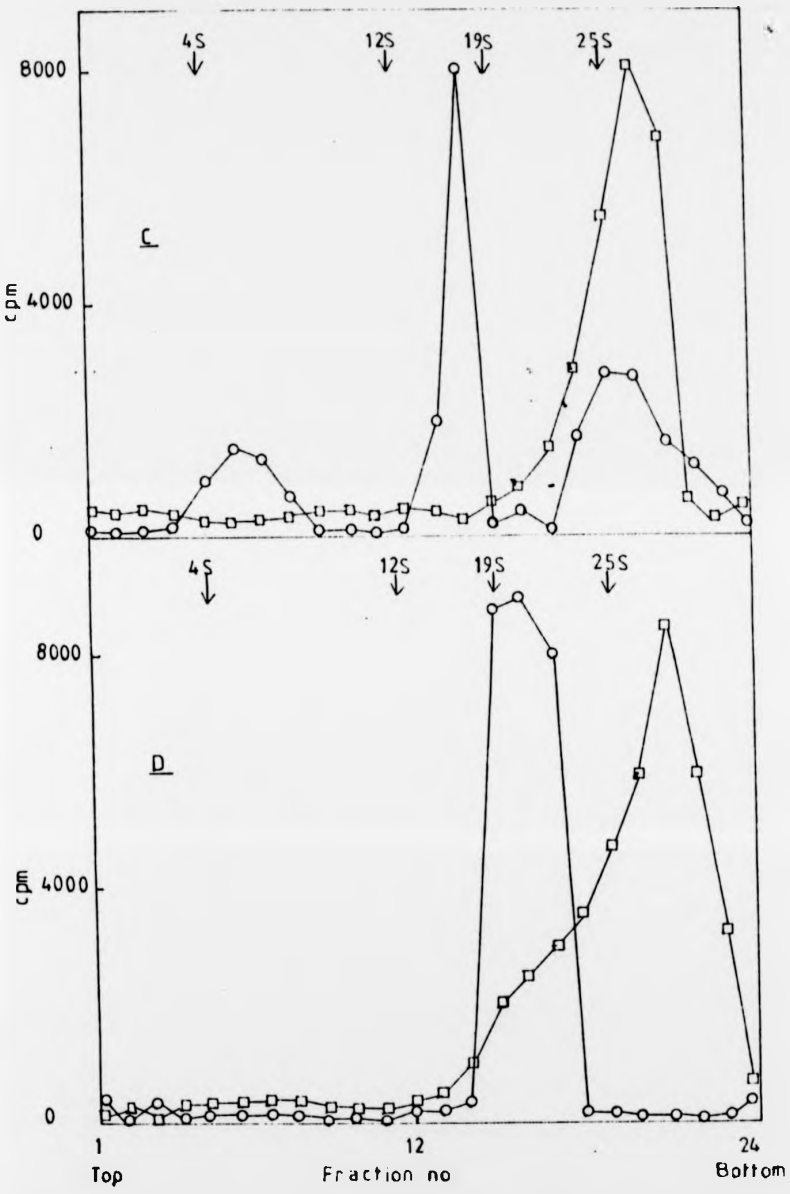



Fig 6.4b Alkaline sucrose density gradient centrifugation of the products of Namalwa cell DNA synthesis after a 24 hour preincubation with $^{14}$C-thymidine (□) followed by a 1 hour pulse of $^{3}$H-thymidine (○), C, in the presence of $10^{-5}$M phosphoramide mustard and D, in the presence of $10^{-5}$M hydroperoxycyclophosphamide. Gradient analysis was carried out as described in 4.2.3, sedimentation is from left to right.

The control incubation was not performed at the same time as the incubations with the alkylating agents as three gradients was the maximum number that could be run at any one time in the rotor used. Because of this a quantitative comparison between the four gradient profiles shown was not carried out as it was decided that this would not be valid under the circumstances (see 4.3.1).

## 6.4 DISCUSSION

The three alkylating agents have different effects on thymidine incorporation by Namalwa cells. Cyclophosphamide has little effect. This is to be expected because the drug has to be metabolised by oxidative enzymes in order to produce the activated, hydroxy, form of the drug. Phosphoramide mustard is an active alkylating agent and therefore inhibits the incorporation of $^{3}$H-thymidine into acid precipitable material as shown in Fig. 6.3. The synthetic derivative of cyclophosphamide, hydroperoxycyclophosphamide, has an even greater inhibitory effect on thymidine incorporation than phosphoramide mustard. The hydroperoxy compound rapidly decomposes in water to give the activated, hydroxy, from of cyclophosphamide. This is then slowly degraded to phosphoramide mustard and acrolein. However the hydroperoxy compound has a greater net effect than phosphoramide mustard on its own. This implies that the action of the drug is not a simple alkylating effect by the phosphoramide mustard formed as a result of its degradation. The synthetic derivative must have other effects in order to produce the observed response. The hydroperoxy compound may be metabolised to other derivatives which may themselves act as alkylating agents or may be more easily transported across the Namalwa cell membrane than phosphoramide mustard (see 6.1). The concentrations of all the drugs required to produce an inhibitory effect is much greater than those concentrations of araC and aphidicolin required to inhibit thymidine incorporation. This is probably

because of the non-specific action of the alkylating agents compared with the specific DNA synthesis inhibitors.

It is thought that the alkylating agents can cross-link double stranded DNA and in this way prevent DNA replication. Cyclophosphamide appears to slow down the rate of DNA elongation. This is indicated by the accumulation of low molecular weight intermediates, 4 S and 12 S, after the sedimentation analysis of the DNA produced by Namalwa cells in the presence of $10^{-3}$ M cyclophosphamide (see Fig. 6.4a). However the inhibition of DNA synthesis, even at this high concentration, is not very significant.

Phosphoramide mustard inhibited DNA elongation as indicated by the accumulation of low molecular weight DNA intermediates. Another effect was also observed, this was the accumulation of a 19 S DNA intermediate. When the Namalwa cells were incubated with $10^{-3}$ M hydroperoxycyclophosphamide the only DNA formed was in the 19 S peak. This unusual peak is probably the result of the cross-linking of DNA by the alkylating agents and this may explain the formation of this unusual class of DNA intermediate. The cross-linking of DNA would produce DNA intermediates of an unusual size and would also prevent the strands from separating on the gradients as is normally the case on alkaline sucrose gradients. The broadening of the $^{14}C$-thymidine 25 S peak may also be a result of the alkylation of preformed 25 S DNA. However before such a hypothesis could be established further, more detailed, experiments should be performed in order to establish that binding of the alkylating agents to DNA occurs in this fashion (see General Discussion).

# CHAPTER 7

## THE IMMUNOSUPPRESSIVE ACTIVITY OF GLUCOCORTICOIDS:- *in vivo* STUDIES

### 7.1 INTRODUCTION

Steroids make an important contribution to the control and regulation of the body's metabolism in their role as naturally occurring hormones. They are secreted by a number of tissues throughout the body and serve a number of functions. The major classes of steroid hormones are (1) the mineralocorticoids produced by the adrenal cortex which are responsible for regulating sodium levels in the body, (2) the oestrogens produced by the ovaries, (3) progesterone also produced by the ovaries and placenta and (4) the androgens produced by the testes and adrenal cortex, all of which control sexual development and reproduction, and finally, (5) glucocorticoids produced by the adrenal cortex which help regulate carbohydrate, protein and lipid metabolism and also mediate in inflammatory responses.

The glucocorticoids and their derivatives have two important pharmacological properties, they act as immunosuppressants and also possess anti-inflammatory activity. The former property is used clinically in the suppression of the T lymphocyte mediated rejection of tissue grafts and organ transplants. The latter property is used clinically in the treatment of a number of diseases associated with the inflammation of tissues including psoriasis and rheumatoid arthritis. The action of the glucocorticoids and their chemically synthesised derivatives are investigated in the following two chapters using an *in vivo* and *in vitro* assay system. This part of

the work was carried out predominantly at Upjohn Limited as part of their study on topical anti-inflammatory agents.

## 7.2 THE MECHANISM OF ACTION OF GLUCOCORTICOIDS

Glucocorticoids produce a number of diverse responses depending upon the target tissue for the steroid (144). In many tissues such as muscle they stimulate catabolism whereas in other tissues they stimulate anabolism. In this respect they can be viewed as preparing the body for stress situations. Thus there is an inhibition of glucose uptake and utilisation in lymphoid cells, skin, connective tissue and adipose tissue. In these tissues and muscle there is a general increase in catabolism affecting carbohydrate, protein, fat and nucleic acid metabolism leading sometimes to cell death or growth inhibition. Other tissues such as brain, heart, liver and erythrocytes are spared this increase in catabolism and are capable of using the increased glucose that is produced.

The glucocorticoids also play a role in the control of the cardiovascular process. In many cases their actions are permissive, i.e. certain cyclic AMP mediated hormonal effects require the presence of the glucocorticoid hormones (144, 145). They also induce several enzymes such as tryptophan pyrrolase and tyrosine transaminase in the liver.

Their inhibitory effect on the metabolism of lymphoid cells resulting in lymphoid cell death is the direct cause of their immunosuppressive effect. Human lymphocytes are relatively resistant to glucocorticoid inhibition unlike mouse and rat lymphocytes. The latter have therefore been extensively used in experimental studies on immunosuppression. The major lymphoid targets are the T lymphocytes. In some instances humoral immunity is also affected though in some

cases this may be an indirect effect due to the inhibition of T helper cell activity (146). The sensitivity of lymphoid cells to glucocorticoids changes during lymphocyte development (144). Both mature B and T lymphocytes appear to be sensitive to glucocorticoids but lose this susceptibility when stimulated by an antigen to undergo antigen induced differentiation. T lymphocyte progenitor cells are also resistant, therefore there appears to be a resistant-sensitive-resistant development sequence, at least for T lymphocytes.

Glucocorticoids have a pronounced effect on white blood cell distribution (146). They act on neutrophils by causing their release from bone marrow stores, prolonging their half-life in the circulation and blocking their migration and accumulation in inflammatory loci. This may be due to the inhibition of lymphokine production by T lymphocytes. This mechanism also prevents the accumulation of macrophages at inflammatory loci. The prevention of cellular accumulation at sites of inflammation is probably one of the major anti-inflammatory effects of glucocorticoids.

Another possible explanation for their anti-inflammatory activity is their inhibitory effect on prostaglandin biosynthesis. Prostaglandin release is a contributory factor in the cause of inflammation. The steroids act by preventing the release and/or activation of the enzyme phospholipase $A_2$. This enzyme releases fatty acids from phospholipids thus providing the precursors for prostaglandin biosynthesis so its inhibition results in a lack of substrates for prostaglandin biosynthesis (147, 148).

There is a unifying hypothesis which explains the mechanism of action behind these diverse responses to glucocorticoids (145). This hypothesis states that the steroids pass through the cell membrane and enter the cytoplasm where they are bound by cytoplasmic receptor

proteins. The receptor proteins exist in a state of equilibrium between a form which will not and one that will ultimately bind in the cell nucleus (144). Active glucocorticoids influence a shift in this equilibrium to a form that can spontaneously undergo a second activation step that exposes a nuclear binding site on the receptor. This steroid receptor complex then binds to the chromatin within the nucleus. The binding of the steroid-receptor complex to nuclear chromatin results in the stimulation of RNA transcription. It is thought that the steroid receptor is a dimeric protein. One of the monomers is thought to bind to a specific non-histone protein on the chromatin. The dimer then dissociates and the other monomer interacts with DNA enabling RNA polymerase to associate with an initiation site on the DNA molecule so that a segment of the DNA can be transcribed. Ribosomal and transfer RNA synthesis are altered depending on the target tissue. There is also an increase in messenger RNA synthesis. This is reflected by increases in both RNA polymerases I and II. These changes lead to the synthesis of specific proteins within the tissue which in turn produce the observed metabolic changes. There is a great deal of experimental evidence to support this hypothesis though much of the detail of the cytoplasmic and nuclear events still has to be elucidated.

## 7.3 STEROID STRUCTURE-ACTIVITY RELATIONSHIPS AND THEIR ANTI-INFLAMMATORY EFFECTS

It is useful when designing a series of drugs based on one basic structure to correlate drug activity with the structural requirements needed to produce that activity. Steroids have to bind to cytoplasmic receptors in order to elicit an effect so a study of the steroid structure in relation to receptor binding is one method of correlating structure with activity (149). Another useful method is to correlate

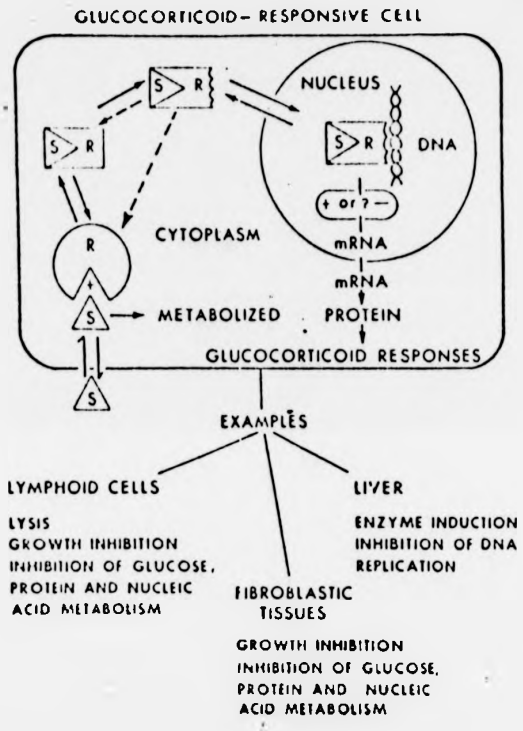



Fig 7.1 The mechanism of action of glucorticoids. S represents the steroid and R its cytoplasmic receptor. Different shapes of R represent different conformational states. The dotted line indicates that it is not known how the activated R-S complex dissociates.

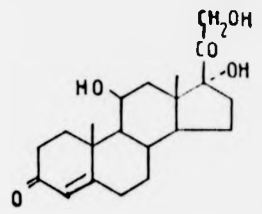
structure with activity using an *in vitro* assay system. One such system involves measuring the degree of induction of the enzyme tyrosine transaminase in rat hepatoma cells. A theoretical approach to structure is also useful, based on such techniques as NMR, X-ray diffraction and energy optimisation by calculating internal strain relaxation in different steroid derivatives (150, 151). This allows a correlation to be made between the three-dimensional structure of the steroid molecule and its effect on a target tissue. The binding of a steroid to its cytoplasmic receptor is dependent upon its spatial arrangement. The biochemical response produced by a steroid is also determined by its three-dimensional structure. Different substituents can radically alter the steric arrangement of the rings of the steroid molecule and radically alter its effect on a target tissue. A theoretical study of three-dimensional structure can therefore be used to predict the cells response to a particular steroid.

Another important structural consideration is based upon the clinical requirements of the drug. Drugs that are to be used topically for the treatment of localised inflammations have to be lipophilic so that they can be readily absorbed by the skin. Topical drugs also must ideally have a large local effect and very little systemic activity, i.e. the drug should have a maximum effect at the site of application rather than at a site removed from the site of application (152).

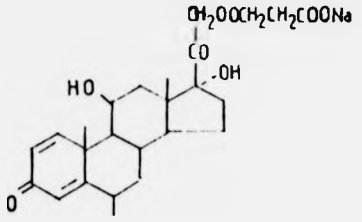
As a result of such studies a number of structural requirements which can enhance the pharmacological activity of steroids have been discovered. Receptor binding is enhanced by a 17 α-OH group and an 11 β-OH group, whereas an 11 α-OH group decreases receptor affinity. The pharmacological activity of cortisol can be increased by a double bond between C-1 and C-2, by 2α and 6α-$CH_3$ groups and halogens

substituted for hydrogen at C-6 and C-9. In the case of topical steroids substituents, usually esters, at C-17 increase their lipophilicity, and hence their bioavailability, thus giving an increased local pharmacological effect (153). The structures of typical glucocorticoids are shown in Fig. 6.2. These are (1) the physiological glucocorticoid, cortisol, (2) 6-α-methyl prednisolone which is used as an immunosuppressant in transplant surgery and as an anti-cancer drug, and the topical steroids, (3) clobetasol propionate and (4) clobetasone butyrate.

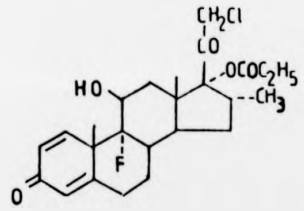
At Upjohn Limited an animal model assay system, in which I assisted, has been developed to test potential topical drugs. The assay system makes use of female hairless mice. The mice are treated with the drug which is spread over 3 $cm^2$ of the anterior dorsal surface (neck) of the animal. The animal is then injected with 25 μCi $^3$H-methyl thymidine subcutaneously in the right thigh. The animals are killed one hour later using ether and skin samples removed from the neck and the left side of the back. The epidermis is then removed by placing the sample, dermis down, on a stainless steel plate at 57°C for 25 seconds after which the epidermis can be easily removed from the dermis with a scalpel blade. The epidermal samples are then sonicated in 0.24 M phosphate buffer pH 6.8 containing 8 M urea, 1% sodium lauryl sulphate and 1 mM EDTA. The DNA is then extracted from the sonicates by column chromatography on hydroxyapatite columns. The columns, consisting of 1 g of hydroxyapatite, are washed with 0.2 M phosphate buffer pH 6.8 containing 8 M urea. The epidermal lysates are applied to the columns and RNA and protein removed by washing with 50 mls of the same buffer. Urea is then removed by washing with 50 ml 0.14 M phosphate buffer pH 6.8. The double stranded DNA, which binds to the columns under conditions of low salt concentration, is then eluted with 0.48 M phosphate buffer pH 6.8 and



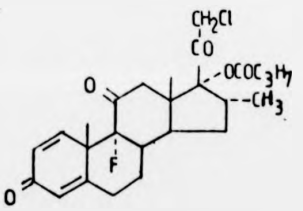

1) Cortisol




2) 6-α-methyl prednisolone 21 sodium succinate




3) Clobetasol propionate




4) Clobetasone butyrate

Fig 7.2 The structures of cortisol and analogues used in the experimental studies described.

a 2 ml sample collected.

The DNA specific activity of the samples is determined by measuring the DNA concentration using UV absorption at 260 nm. A concentration of 50 µg/ml DNA has an absorbance of one. The degree of radioactive thymidine incorporated into the DNA is determined by adding 0.5 ml of the sample to 12 mls scintillant cocktail (3 parts PCS (Searle): 2 parts toluene) and 0.2 ml M HCl in scintillation vials and the radioactivity measured in a scintillation counter. Epidermal DNA synthesis activity is expressed as dpm/µg DNA.

This method has been used to compare the local and systemic effects of topically applied steroids by comparing the degree of inhibition of local and systemic (neck and back respectively) epidermal DNA synthesis by the drugs. Using this system it was found that clobetasol propionate has both a marked local and systemic effect. Clobetasone butyrate has negligible systemic effect but a good local effect, though to a lesser extent than clobetasol propionate (152, 154). These results suggest that the modification at the C-11 position on the steroid with either an alcohol or keto group can profoundly alter the pharmacological activity of the steroid. Using these studies as a basis for further work it was decided to carry out further studies into the anti-inflammatory effects of these glucocorticoids.

## 7.4 THE DELAYED-TYPE HYPERSENSITIVITY REACTION

The term delayed-type hypersensitivity (DTH) was first applied to a group of cutaneous hypersensitivity reactions that showed a delayed response to a specific antigen. The response occurs on re-exposure to the antigen after the previous sensitisation of the cellular immune system to that antigen. The types of antigen that elicit the DTH response are proteins or protein-hapten complexes, e.g. tuberculin,

which are injected subcutaneously, or topically applied contact sensitising agents such as picryl chloride or oxazolone (see Fig. 7.3).

The DTH response is primarily a T lymphocyte mediated response, these being the major effector cells, though there is some evidence that a type of B suppressor cell may play a role in the regulation of the DTH response (155, 156). The DTH response can be transferred by the passive transfer of lymphocytes from a sensitised animal to an unsensitised animal. The latter will then exhibit a DTH response to a challenge by the antigen thus confirming that DTH is a cell-mediated immune response rather than a humoral immune response (157, 158).

The DTH response is characterised by a local cellular infiltration of lymphocytes and macrophages which results in oedema and inflammation (157). These phenomena are directly caused by lymphokines produced by the lymphocytes. Macrophage migration is inhibited by MIF leading to macrophage accumulation (9). The lymphokines also cause increased vascular permeability and promote oedema. The delayed-type hypersensitivity response exhibits circadian rhythmicity probably due to the variations in circulating lymphocyte numbers thoughout the day (160, 161, 162). In man the lymphocyte count is high during the day and decreases at night. In nocturnally active rats the lymphocyte count increases at night and decreases during the day. This may prove to be an important consideration when scheduling drug regimens.

The DTH response can be produced in the laboratory using rats or mice sensitised with oxazolone (157, 163). It can be used as a model system for testing immunosuppressive and anti-inflammatory drugs such as the glucocorticoids (164, 165). The principle of the assay involves sensitising the animals with oxazolone by applying it to the shaved

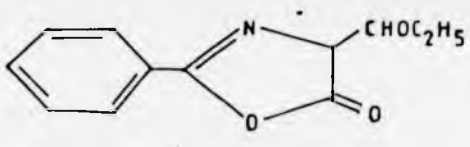



Fig 7.3 Oxazolone

abdomen of the animals. After a period of about nine days a challenge dose of oxazolone is applied to the ear. This challenge dose causes the ear to swell as a result of local oedema and inflammation caused by the DTH response. This increase in ear thickness can be used as a measure of the DTH response. The steroid to be tested can be applied at the same time as the challenge dose of oxazolone and its effect on the DTH response monitored. Steroids given after the challenge have little or no effect on the DTH response (164).

## 7.5 MATERIALS AND METHODS

### 7.5.1 Materials

Oxazolone (4-ethoxymethylene-2-phenyl oxazolone) was obtained from BDH Chemicals Limited. Clobetasol propionate and clobetasone butyrate were obtained from Glaxo, 6α-methyl-prednisolone-21-sodium succinate (Solu-medrone) was obtained from Upjohn Limited. The animals used were male inbred CBA mice (Olac 1976), 30-35 g weight. The animals were housed in a room with a light cycle of 10 a.m. to 10 p.m. for at least two weeks prior to and during the study.

### 7.5.2 The delayed-type hypersensitivity assay

The procedure used for this assay was adapted from the method of Asherson and Ptak (157). On the morning of Day 1 the animals were anaesthetised with ether and their ventral abdominal region shaved with electric clippers. The animals were sensitised to oxazolone by applying 100 µl of 10% oxazolone in a 1:1 mixture of ethanol:acetone over 5 $cm^2$ of the abdomen. At approximately the same time on Day 8 the mice were again anaesthetised and the thickness of their left ears measured with an engineers micrometer screw guage, three measurements were taken each time and the mean determined. Following this a 20 µl challenge dose of 10% oxazolone in a 4:1 mixture of

acetone:olive oil was applied to the left ear and the change in thickness of the left ear measured at regular intervals up to 48 hours after challenge.

### 7.5.3 The effect of glucocorticoids on the delayed-type hypersensitivity response

6-α-methyl prednisolone, which is water soluble but insoluble in organic solvents and therefore unsuitable for topical application, was dissolved in physiological saline and administered by an intramuscular injection at a dose of 14 mg/kg. The steroid was administered at the same time as the application of the challenge dose of oxazolone. The change in ear thickness was measured at 6, 12, 24 and 48 hours after oxazolone challenge.

The effect of the two topical steroids on the DTH response was determined by applying 20 µl of the steroids at a concentration of 0.05% in ethanol to the left ear of the animals at the same time as the oxazolone challenge dose. The control animals were similarly treated with 20 µl ethanol. The change in ear thickness was measured at 6, 12, 24 and 48 hours after oxazolone challenge.

The effect of increasing doses of clobetasol propionate, the most effective of the two topical steroids, was investigated. This was carried out by applying 20 µl of 0.001, 0.01, 0.05 and 0.2% steroid in ethanol to the left ear of the animals at the same time as the challenge dose of oxazolone. The control animals were similarly treated with 20 µl ethanol. The change in ear thickness was measured at 12 and 24 hours after challenge. Two other steroids synthesised by the Upjohn company were also tested using this assay. However due to the restrictions placed upon employees in industry these results must remain confidential and are therefore not shown.

## 7.6 RESULTS

### 7.6.1 The effect of 6-α-methyl prednisolone on the DTH response in mice

Within the first 12 hours the intramuscular injection of 6-α-methyl prednisolone markedly inhibited the extent of ear swelling in the mice caused by the oxazolone challenge (see Fig. 7.4). The degree of inhibition of ear swelling decreased after that time so that at 24 hours after challenge the change in ear thickness in the control and steroid treated animals is approximately the same. At 48 hours after challenge the ear thickness of both sets of animals had decreased. However the decrease in ear thickness was greater in the steroid treated mice than in the control mice.

### 7.6.2 The effect of topically applied steroids on the DTH response in mice

Both clobetasol propionate and clobetasone butyrate inhibited the extent of the ear swelling caused by the oxazolone challenge (see Fig. 7.5). However the degree of inhibition of ear swelling caused by clobetasol propionate is greater than that caused by clobetasone butyrate. The former inhibited the extent of ear swelling by 70% whereas the latter inhibited the extent of ear swelling by 40% at 12 hours after oxazolone challenge. The ear thickness in the control and experimental animals decreased 12 hours after challenge but the ratios of change in ear thickness between the three groups of animals remained approximately the same throughout the 48 hours that measurements were taken.

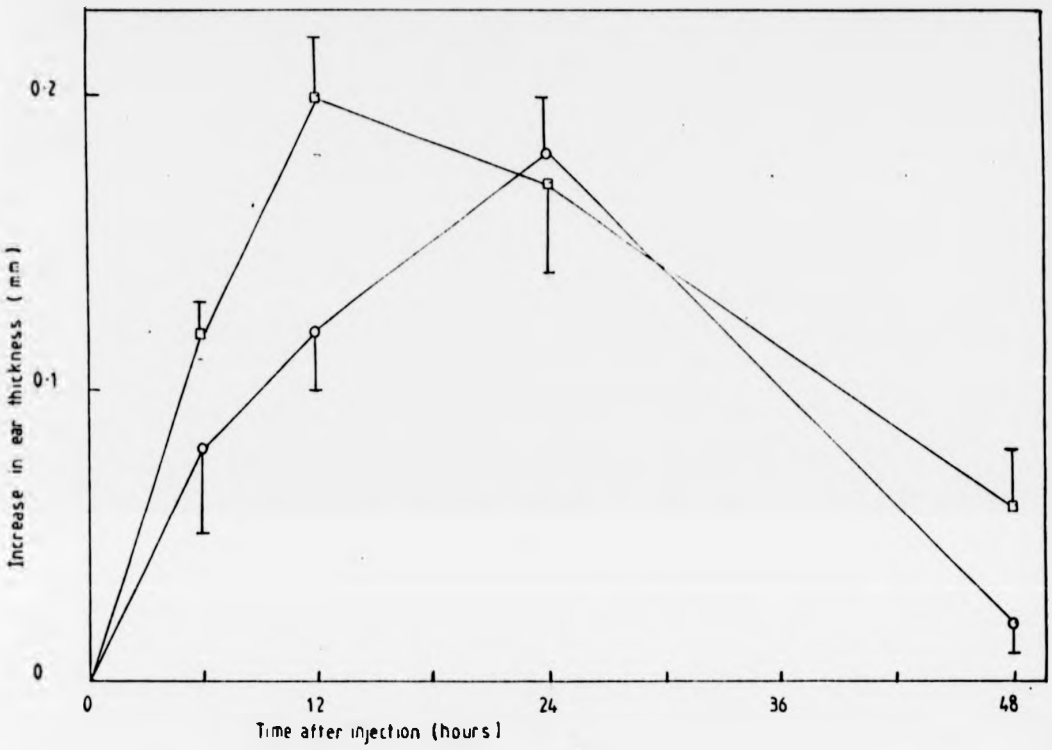



Fig 7.4 The effect of 6-α-methyl prednisolone administered by an intramuscular injection on the DTH response in CBA mice as measured by the subsequent increase in ear thickness after oxazolone challenge. (□)control, (○) 6-α-methyl prednisolone (i.m.). (14mg/kg) error bars represent S.E. n=5.

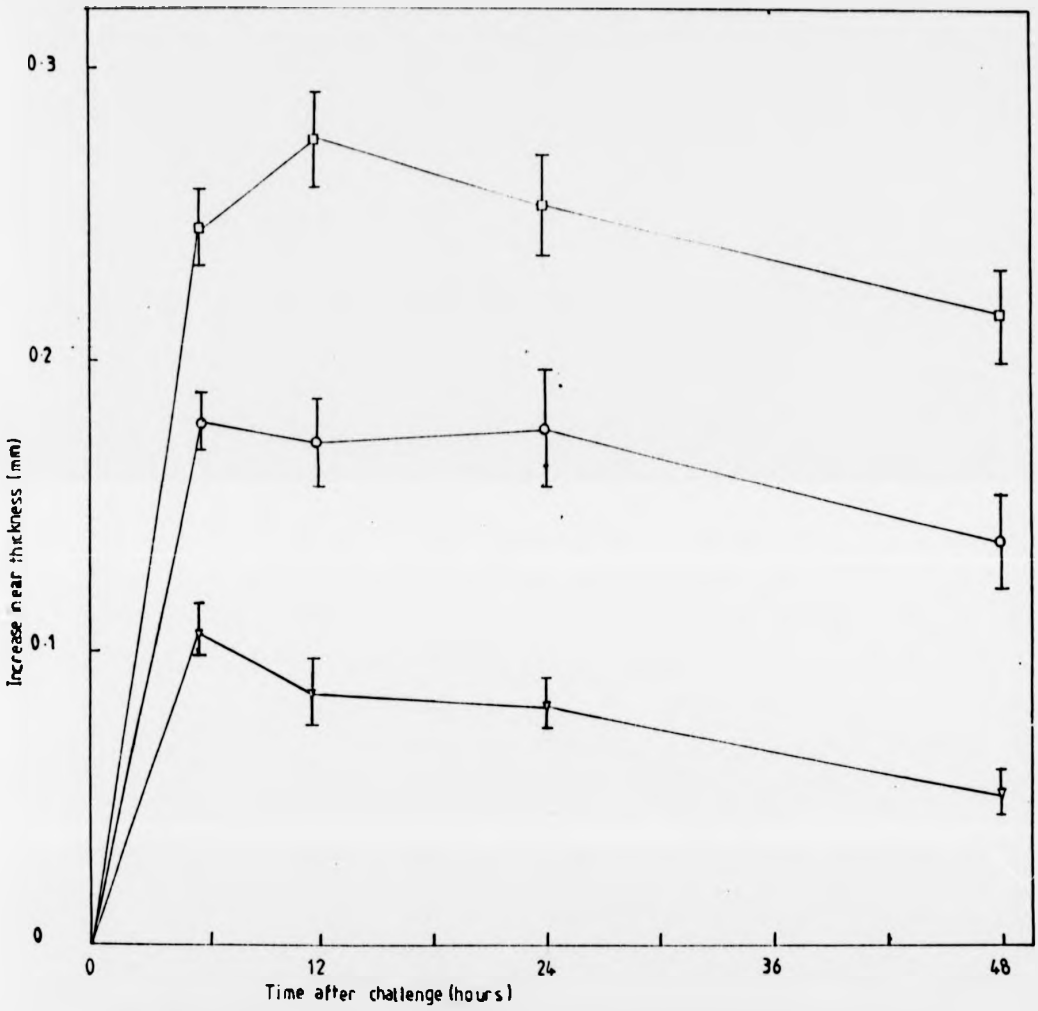



Fig 7.5 The effect of topically applied steroids (20ul) on the DTH response in CBA mice as measured by the subsquent increase in ear thickness after oxazolone challenge.(□) ethanol (control), (▽) 0.05% clobetasol propionate,(○) 0.05% clobetasone butyrate. Error bars represent S.E. n=4

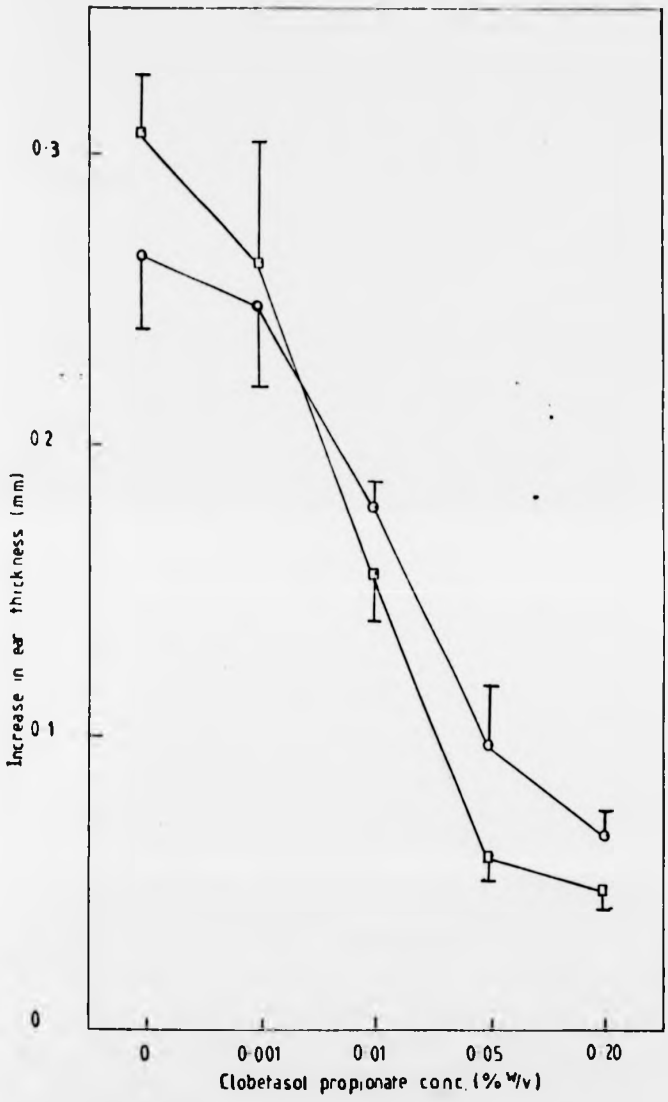



Fig 7.6 The effect of increasing doses of clobetasol propionate on the DTH response in CBA mice as measured by the subsequent increase in ear thickness (o)12 hours after oxazolone challenge and (□)24 hours after oxazolone challenge.
Error bars represent S.E. n=9

### 7.6.3 The effect of increasing doses of clobetasol propionate on the DTH response in mice

The graphs in Fig. 7.6 show two things. The first is that the degree of inhibition of ear swelling by clobetasol propionate is approximately the same, within the limits of the errors, at 12 and 24 hours after oxazolone challenge independent of the steroid concentration. The second is that the degree of inhibition of ear swelling increases with increasing doses of steroid so that though there is almost maximum ear swelling with 0.001% clobetasol propionate ear swelling has been almost completely inhibited by 0.2% clobetasol propionate.

## 7.7 DISCUSSION

The glucocorticoids can act both as immunosuppressants and anti-inflammatory agents. Their anti-inflammatory effect has been demonstrated by their ability to suppress epidermal DNA synthesis in hairless mice. The work in this chapter has also shown that they are capable of inhibiting the inflammatory response to the contact sensitising agent oxazolone caused by the delayed-type hypersensitivity reaction. In some cases the immunosuppressant and anti-inflammatory effects of steroids appear to be unconnected. However in the case of the inhibition of the DTH reaction the anti-inflammatory action of the steroids is probably a direct result of the suppression of cell-mediated immunity by the drugs, the DTH reaction being predominantly a T lymphocyte mediated response.

The water soluble steroid, 6-α-methyl prednisolone, exhibits an inhibitory effect on the DTH response when administered by intra-muscular injection but its effect is not as great as that exhibited by the topical steroids. The most obvious explanation for this is that 6-α-methyl prednisolone could not be administered locally but

had to reach the site of action via the systemic circulation. Because of this it could not be concentrated at the site of the inflammation like the topical steroids but would have been distributed throughout the animal's body. Another possible explanation is that 6-α-methyl prednisolone does not have a fluorine group at C-9 like the topical steroids. The presence of a halogen at C-9 has been shown to increase the pharmacological activity of the glucocorticoids. However this probably has only a minor effect as 6-α-methyl prednisolone is a known, and clinically used, inhibitor of cell-mediated immunity, though this assumes that the observed anti-inflammatory effect of the steroids in the DTH assay is directly connected with their immunosuppressant activity.

The hairless mouse assay has shown that both clobetasol propionate and clobetasone butyrate possess good anti-inflammatory activity. Clobetasol propionate inhibits local epidermal DNA synthesis in this system to a greater extent than clobetasone butyrate. However this drug also gives a pronounced systemic effect whereas this is negligible with clobetasone butyrate. This effect has been shown to be directly associated with the presence of the keto group at C-11 rather than the alcohol group. Because of the lack of systemic activity clobetasone butyrate is the better topical drug as its effect is localised to the site of application.

The greater effect of clobetasol propionate is again demonstrated in the DTH assay. Clobetasol propionate inhibits the ear swelling caused by the DTH response to oxazolone more effectively than clobetasone butyrate. This assay however gives no indication of systemic activity. The inhibitory effect of clobetasol propionate has been shown to be dose dependent, the degree of inhibition increasing as drug dose increases.

When designing an anti-inflammatory drug for topical application two important considerations have to be borne in mind. The potential drugs have to be selected so that they have maximum local effect and minimum systemic effect. They must also be selected for their anti-inflammatory effect rather than their immunosuppressive effect. Continued use of steroids that depress the immune response can lead to secondary infections of the lesions. By using more than one assay system for investigating the pharmacological activity of steroids these parameters can be compared with respect to steroid structure-activity relationships and the ideal drug selected.

# CHAPTER 8

## THE IMMUNOSUPPRESSIVE ACTIVITY OF GLUCOCORTICOIDS: *in vitro* STUDIES

### 8.1 ISOLATED LYMPHOCYTES AS A MODEL SYSTEM

The DTH reaction discussed in Chapter 7 is a lymphocyte mediated response. Lymphocytes can be easily isolated from lymphoid tissues and blood and used to provide a useful *in vitro* analogue of the *in vivo* immunological assays. They also provide a useful system for the study of a number of biochemical events such as DNA (103, 166, 167), RNA (168) and protein synthesis (169). They have also been used extensively in the study of leukaemia (117, 170, 171).

Isolated lymphocytes can be stimulated *in vitro* to undergo mitosis and differentiation to blast cells by a variety of chemical substances known as mitogens. These bind to the glycosidic groups of glycoproteins in the lymphocyte membrane and elicit a number of biochemical responses leading to blast formation. Some mitogens specifically stimulate one lymphocyte sub-population whereas others can stimulate both B and T lymphocytes (172). The most commonly used mitogens are the plant lectins, phytohaemagglutinin (PHA) and concanavalin A (conA). Other commonly used mitogens are pokeweed mitogen (PWM) and bacterial lipopolysaccharides (LPS). ConA and PHA selectively stimulate T lymphocytes whereas LPS selectively stimulates B lymphocytes, PWM stimulates both B and T lymphocytes. As well as inducing blastogenesis, mitogens can also induce B and T cell responses. For example PHA will induce lymphokine production in T cells and PWM will induce IgM secretion by B cells (173). The PHA response can also be modulated

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by the presence of different populations of cells in the culture such as suppressor T lymphocytes and monocytes (174, 175).

The mitogen used in the present studies was PHA, a protein isolated from the red kidney bean *Phaseolus vulgaris*. Being an activator of T lymphocytes this should provide an analogous system to the DTH assay which is a T lymphocyte mediated response. There are four separate steps in lymphocyte activation by PHA (176). These are (1) attachment to the cell membrane, (2) a reversible pre-activation step, (3) a step causing commitment to commence mitosis and (4) a mitogen-independent activation step. After three days of exposure to PHA isolated lymphocytes form blast cells, most of which are starting to undergo mitosis (177). The nucleus in these blast cells is larger than the nucleus in the precursor lymphocytes and this correlates with an increase in DNA synthesis (178). There are marked nucleolar changes four hours after exposure to PHA. There is an increase in nucleolar size followed by an increase in the amount of nucleolar chromatin. The nucleolar chromatin becomes more dispersed and is found concentrated on the nuclear membrane and throughout the nucleoplasm. 48-72 hours after exposure to PHA there is an increase in cytoplasmic size and in the number of cytoplasmic components, e.g. lysosomes. With some mitogens, e.g. PWM the cells are characterised by a well developed rough endoplasmic reticulum and Golgi apparatus. However with PHA blasts though there are well formed Golgi bodies there is sparse rough endoplasmic reticulum and many free ribosomes (179, 180).

The increase in DNA synthesis upon PHA stimulation can be easily measured by the incorporation of radioactive thymidine into acid precipitable material. The increase in DNA synthesis corresponds to an increase in DNA polymerase $\alpha$ levels three days after exposure to

PHA (181, 182). The rate of DNA synthesis then decreases. A second wave of DNA synthesis has been reported 5-8 days after exposure to PHA (183, 184). This corresponds to an increase in DNA ligase and DNA polymerase β levels and may represent DNA repair. Prior to the onset of DNA replication there is an increase in RNA and protein synthesis (182). This implies that blast activation and the corresponding increase in DNA synthesis first requires the synthesis of new protein including the enzymes involved in DNA replication. One study suggests that the increase in DNA synthesis is due to an increase in the transport and uptake of precursors of DNA replication by the stimulated lymphocytes rather than by the activation of the DNA replication apparatus but this seems unlikely (103).

Another possible method for stimulating isolated lymphocytes to undergo blastogenesis is to use a hapten-carrier conjugate (185). The stimulation of isolated lymphocytes by an oxazolone-protein conjugate could be used as an analogue of the DTH response *in vitro*. It is thought that in the *in vivo* situation, the oxazolone must bind to a protein in the skin before it can produce a response (186). Oxazolone is too small a molecule to be an antigen in its own right and is therefore thought to behave as a hapten which binds to a large carrier molecule in the skin. This possibility was modelled by using an oxazolone-rat serum albumin conjugate. This was then used for the *in vitro* stimulation of lymphocytes isolated from rat blood thus producing an *in vitro* assay system parallel to the *in vivo* DTH assay system.

The isolated lymphocyte system was used to investigate the effects of glucocorticoids on thymidine incorporation by stimulated lymphocytes. The glucocorticoids in general act in a cell cyclic specific manner being $G_1$/S phase inhibitors (187). Their *in vivo* action is also dependent upon the stage of development of the lymphocytes since they will only inhibit the metabolism of lymphocytes before they are

presented with an antigen (144). Translated to the *in vitro* situation, this means that the steroids will only inhibit the PHA stimulation of lymphocytes if administered in the first 24 hours after stimulation as PHA rapidly induces resistance to steroids (188, 189). It appears as if the glucocorticoids act as PHA antagonists and inhibit the mechanism of PHA stimulated transformation of lymphocytes to lymphoblasts. The inhibition of lymphocyte transformation by steroids is also complicated by the fact that the response to glucocorticoids is modulated and inhibited by interacting cell populations in the lymphocyte cultures such as monocytes and macrophages (190, 191). Steroid receptors also appear to play a part in conferring resistance to steroids upon the transformed lymphocytes. The important factor appears to be the affinity of the steroid for the cytoplasmic receptor (191). It has also been shown that receptor number increases with PHA stimulated transformation. However this seems to be unrelated to the steroid sensitivity of the transformed lymphocyte (192).

This is in contrast to the mechanisms of action of the drugs already studied. A comparative study was carried out using araC and aphidicolin. These drugs, which directly inhibit DNA synthesis, have a maximum effect when administered during the S phase of the cell cycle when the rate of DNA synthesis is at a maximum. Both drugs have been shown to be inhibitors of DNA synthesis in PHA stimulated isolated lymphocytes (103, 135).

## 8.2 MATERIALS AND METHODS

### 8.2.1 Materials

Dextran T-400 and Ficoll-Paque were obtained from Pharmacia Fine Chemicals. Ficoll-Paque is an aqueous solution of density $1.077 \pm 0.001$ g/cm$^3$ containing 5.7 g Ficoll 400 and 9 g sodium

diatrizoate per 100 ml. Phytohaemagglutinin and rat serum albumin were obtained from Sigma Chemical Company. Sheep erythrocytes in Alsever's solution were obtained from Flow Laboratories. All materials required for the culture of isolated lymphocytes are the same as those described in 3.4.1.

### 8.2.2 Isolation of lymphocytes

Human peripheral lymphocytes were isolated from 30 ml or 40 ml blood samples taken from volunteers. The blood samples were collected in EDTA or heparin solution. The blood samples were then diluted 1:1 in sterile bottles with balanced salt solution and 10% Dextran 400 in 0.9% sodium chloride to give a final concentration of 1% Dextran. The blood was then left to stand for at least one hour at 37°C during which time the majority of the red blood cells sedimented to the bottom of the bottle. The sedimentation of the red blood cells is enhanced by dextran. After sedimentation the upper plasma rich layer was removed and centrifuged at 800 g, 10 mins, 18-20°C in a MSE Mistral 6L centrifuge. The supernatant was discarded and the pellet resuspended in a suitable volume of balanced salt solution, this volume being governed by the next step in the procedure.

The lymphocytes were then separated out from the cell suspension by layering the suspension on to Ficoll-Paque. The volume of Ficoll-Paque used depended upon the size of the centrifuge tube used. The relative height of the Ficoll-Paque layer compared to that of the sample layer is critical, the best height ratio was found to be 2.5 cm Ficoll-Paque to 1.5 cm sample. This was then centrifuged at 400 g, 35 mins, 18-20°C. Differential migration through the Ficoll-Paque results in separation of the cells in the suspension. Granulocytes and erythrocytes sedimented to the bottom of the centrifuge tube and the lymphocytes collected at the interface between the two layers (193, 194).

The "buffy" lymphocyte layer at the interface was removed and diluted with three volumes of balanced salt solution and the lymphocytes washed by centrifugation at 100 g, 20 mins, 18-20°C. The lymphocyte pellet was collected and resuspended in RPMI-1640 medium supplemented with L-glutamine, 300 µg/ml, penicillin, 100 U/ml, and streptomycin, 100 µg/ml. The cell viability was determined by the trypan blue exclusion test and the cells counted using a Neubauer haemocytometer. The cells were then diluted to $10^6$ cells/ml and the medium supplemented with 10% new born calf serum and left to equilibrate for 24 hours. PHA was then added to a final concentration of 25 µg/ml of culture.

Rat peripheral lymphocytes were prepared from blood obtained from Wistar rats. The rats were anaesthetised with ether and exsanguinated by withdrawing blood from the inferior vena cava into a heparinised syringe. The blood was then transferred to a sterile 20 ml bottle containing 2 ml heparin solution, 10 U/ml in 0.9% saline. The average volume of blood obtained from a rat was about 10 ml. The blood was then diluted 1:1 with balanced salt solution and 10% dextran added to give a final concentration of 1%. The diluted blood was then left to stand for one hour at 37°C to allow the red blood cells to sediment. The upper plasma rich layer was removed and the lymphocytes isolated on Ficoll-Paque as previously described for the isolation of human peripheral lymphocytes. The lymphocytes were stimulated with either PHA, 25 µg/ml, or the oxazolone-rat serum albumin conjugate.

Lymphocytes were also isolated from rat and mouse spleens. The freshly removed spleens were sliced and then homogenised by passing them through a fine wire mesh and washing with balanced salt solution. The homogenate was left to stand on ice until all the debris had settled. The supernatant was then centrifuged at 800 g, 10 mins, 4°C. The pellet was resuspended in balanced salt solution and the lymphocytes isolated in Ficoll-Paque as previously described.

All glassware used in this procedure was sterile and siliconised. The balanced salt solution was freshly prepared each time from two stock solutions, A and B. Solution A consisted of $5 \times 10^{-5}$ M $CaCl_2.2H_2O$, $9.8 \times 10^{-4}$ M $MgCl_2.6H_2O$, $5.4 \times 10^{-3}$ M KCl, 0.1% glucose and 0.145 M Tris-HCl pH 7.6. Solution B consisted of 0.82% NaCl. The balanced salt solution was prepared by adding 10 ml of solution A to 90 ml of solution B.

### 8.2.3 Sheep erythrocyte rosette test

Human lymphocytes isolated as described in 8.2.2 were tested for their ability to form rosettes with sheep red blood cells. This test was used to determine the percentage of T lymphocytes in the culture (187). 0.1 ml of lymphocytes, $10^6$ cells/ml, was mixed with 0.1 ml sheep erythrocytes in Alsever's solution that had been previously diluted with balanced salt solution to give a suitable distribution of red cells when viewed on a haemocytometer under a light microscope. The suspension was centrifuged at 200 g, 10 minutes at room temperature in a BTL micro-angle bench centrifuge and then incubated for one hour at 20°C in a water bath. The cells were then gently resuspended with a Pasteur pipette and diluted 1:1 with 0.4% trypan blue in 0.9% sodium chloride. The number of rosettes that had formed were counted on a Neubauer haemocytometer. A rosette was defined as a lymphocyte with three or more adherent red blood cells. The percentage of T lymphocytes in the culture was then calculated.

### 8.2.4 Preparation of the oxazolone-protein conjugate

A hapten-carrier conjugate was formed between oxazolone and rat serum albumin in the following way (196). 20 mg oxazolone was dissolved in 1 ml dioxan and added dropwise to 40 ml rat serum albumin solution, 1 mg/ml in 0.05 M sodium carbonate/bicarbonate buffer pH 9. The suspension was well mixed and left to stand overnight at 4°C. It was

then centrifuged at 2000 g, 90 minutes, 4°C and the supernatant dialysed extensively against phosphate buffered saline pH 7.2 in order to remove the dioxan and any unbound oxazolone. The protein concentration of the conjugate was determined by the method described by Lowry *et al.*, using bovine serum albumin as a standard (98). The amount of oxazolone bound to the rat serum albumin was determined by UV spectrophotometry. UV spectra over the range 240 nm to 400 nm were taken for oxazolone, rat serum albumin and the conjugate and these spectra used to determine the amount of bound oxazolone (see 8.3.3).

### 8.2.5 The measurement of $^{3}$H-thymidine incorporation by isolated lymphocytes

1 ml cultures of lymphocytes were incubated with 10 μCi [$^{3}$H-methyl] thymidine sp. act. 47 Ci/mmol and the incorporation of radioactive thymidine into acid precipitable material assayed as described in 3.4.5. The optimal PHA concentration for the stimulation of rat peripheral lymphocytes was determined by measuring $^{3}$H-thymidine incorporation 72 hours after addition of different concentrations of PHA. The period of maximum thymidine incorporation after PHA stimulation in human and rat peripheral lymphocytes was determined by incubating the cells for one hour with radiolabelled thymidine at regular time intervals after stimulation. The effect of the different drugs on thymidine incorporation was determined by incubating the cells for 4 hours with $^{3}$H-thymidine at the period of maximum thymidine incorporation in the presence of the drugs at the required final concentrations. All drugs were dissolved in physiological saline except for aphidicolin which was dissolved in DMSO and added to the cultures so as to give a concentration of DMSO of not greater than 1%. The effect of the oxazolone-rat serum albumin conjugate on $^{3}$H-thymidine incorporation by rat peripheral lymphocytes was investigated. The effect of PHA on thymidine incorporation by rat and mouse spleen lymphocytes was also investigated. All results are

shown as a percentage inhibition of the maximum incorporation of thymidine into acid precipitable material.

## 8.3 RESULTS

### 8.3.1 Thymidine incorporation by PHA stimulated human lymphocytes

The lymphocytes isolated from human blood showed a viability of greater than 90% in most cases. The sheep erythrocyte rosette tests showed that the majority of the lymphocytes isolated using the Ficoll-Paque method were T lymphocytes, generally 90-95% of the isolated lymphocytes were T lymphocytes.

Isolated human lymphocytes were pulsed with $^{3}$H-thymidine for one hour at 11, 23, 35, 47, 59, 71, 83 and 95 hours after the addition of PHA. A control experiment was carried out with lymphocytes which had not been treated with PHA. The results, which are shown in Fig. 8.1, are representative results from a number of similar experiments. The period of maximum thymidine incorporation occurs at 72 hours after PHA stimulation, after which there is a decrease in the ability of the lymphocytes to incorporate thymidine. There is little thymidine uptake in unstimulated lymphocytes.

Human lymphocytes treated with PHA were pulsed with $^{3}$H-thymidine for 4 hours starting 68 hours after the addition of PHA. Methyl prednisolone was added at final concentrations of 0, $10^{-12}$, $10^{-10}$, $10^{-8}$, $10^{-6}$ and $10^{-4}$ M at the same time as the PHA. The effects of the drugs on thymidine incorporation are shown in Fig. 8.2. 37% inhibition is obtained with $10^{-8}$ M methyl prednisolone and 92% inhibition is obtained with $10^{-4}$ M. The experiment was repeated with cytosine arabinoside at final concentrations of 0, $10^{-12}$, $10^{-10}$, $10^{-8}$, $10^{-6}$ and $10^{-4}$ M. In this case the araC was added concurrently with the $^{3}$H-thymidine.

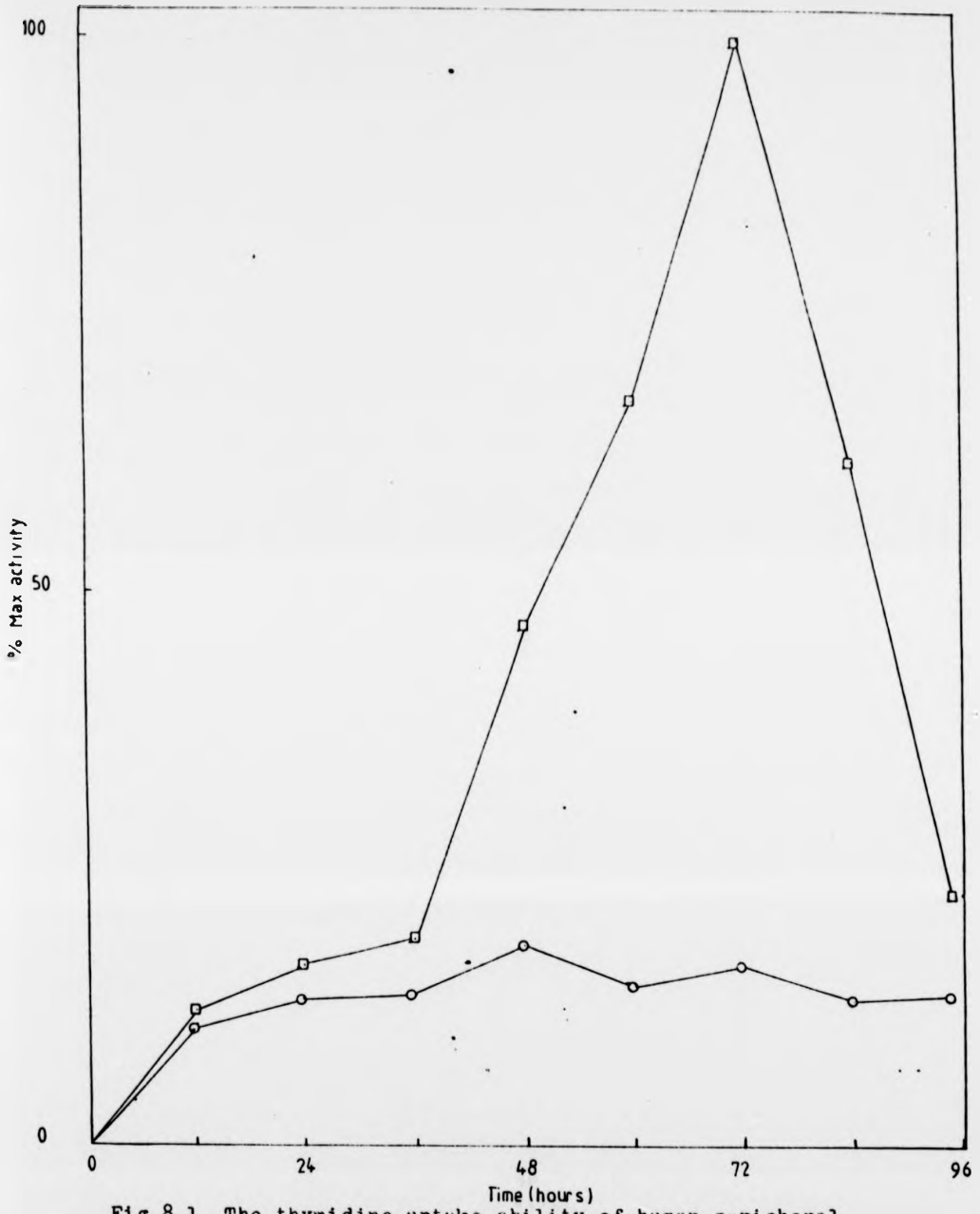



Fig 8.1 The thymidine uptake ability of human peripheral lymphocytes with time after the addition of PHA (25ug/ml) at zero time (□), (○) is a control without PHA. Results are shown as the percentage of the maximum thymidine incorporation into acid percipitable material as described in 3.4.4

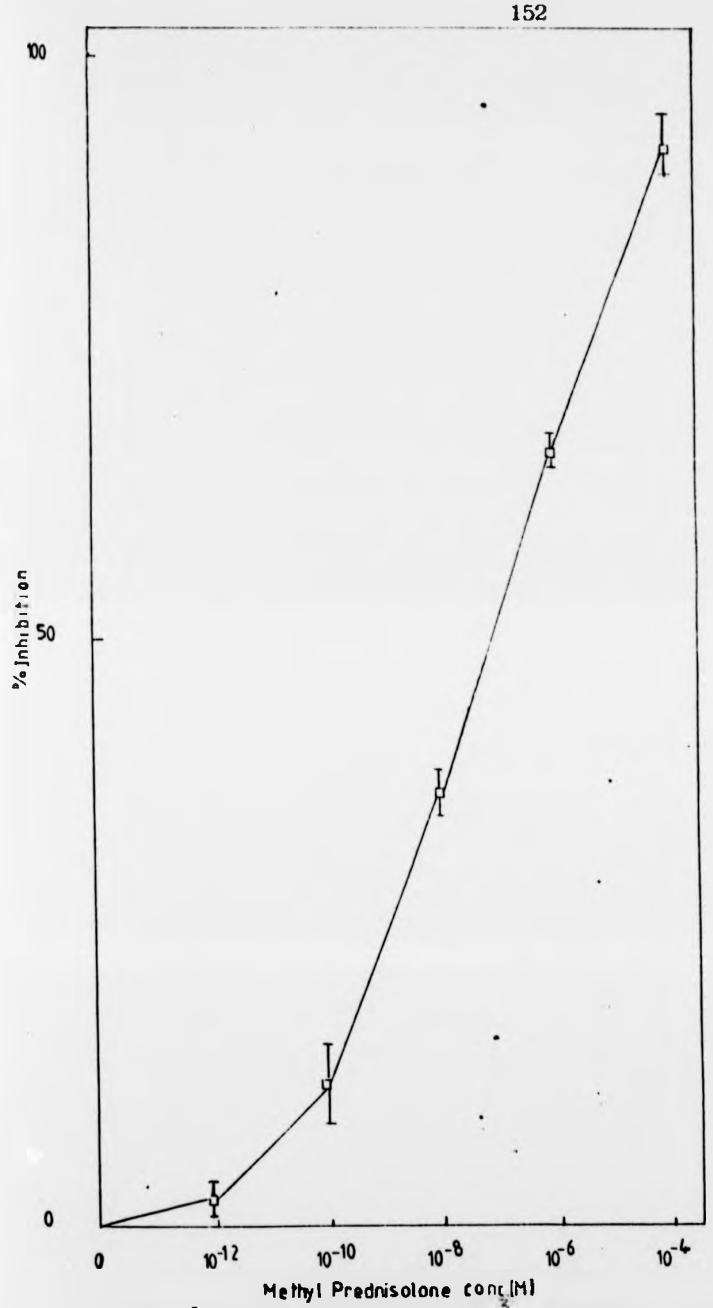



Fig 8.2 The inhibition of $^{3}H$ thymidine uptake in PHA stimulated human peripheral lymphocytes by 6-α-methyl prednisolone. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E. n=4

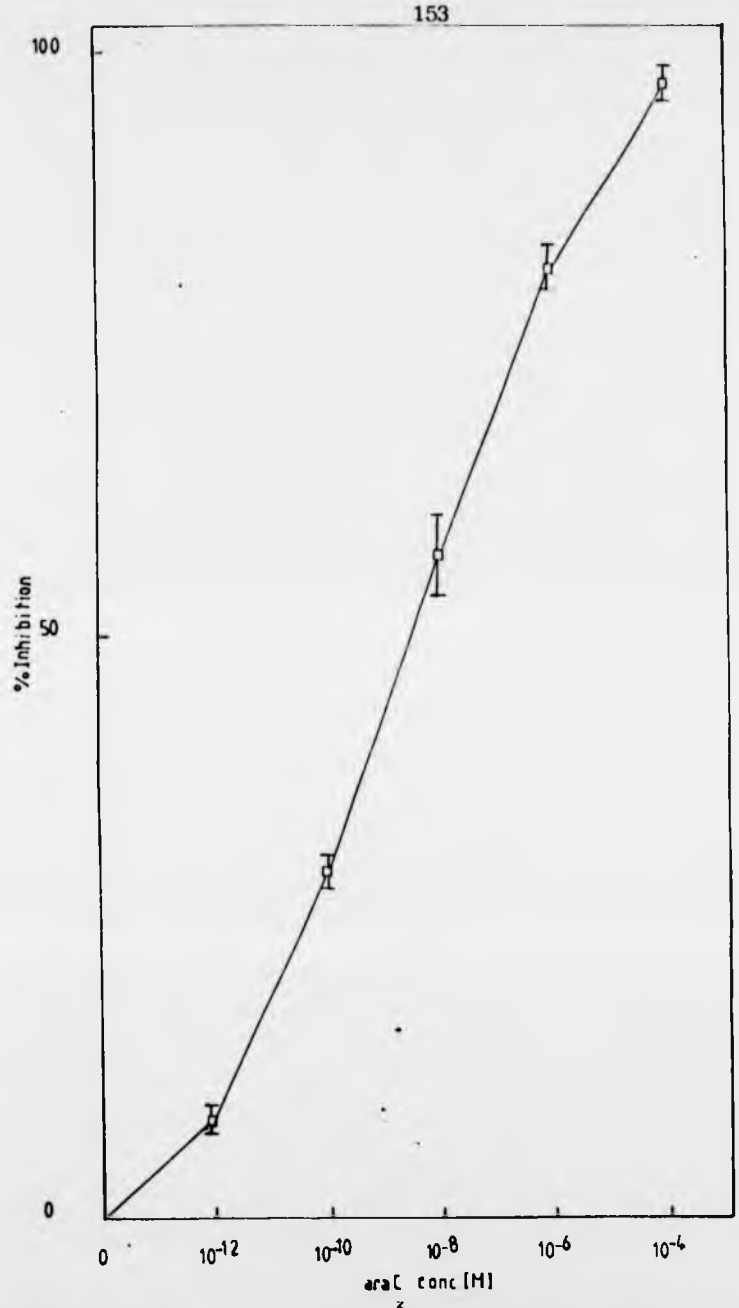



Fig 8.3 The inhibition of $^{3}H$ thymidine uptake in PHA stimulated human peripheral lymphocytes by cytosine arabinoside.. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E. n=4

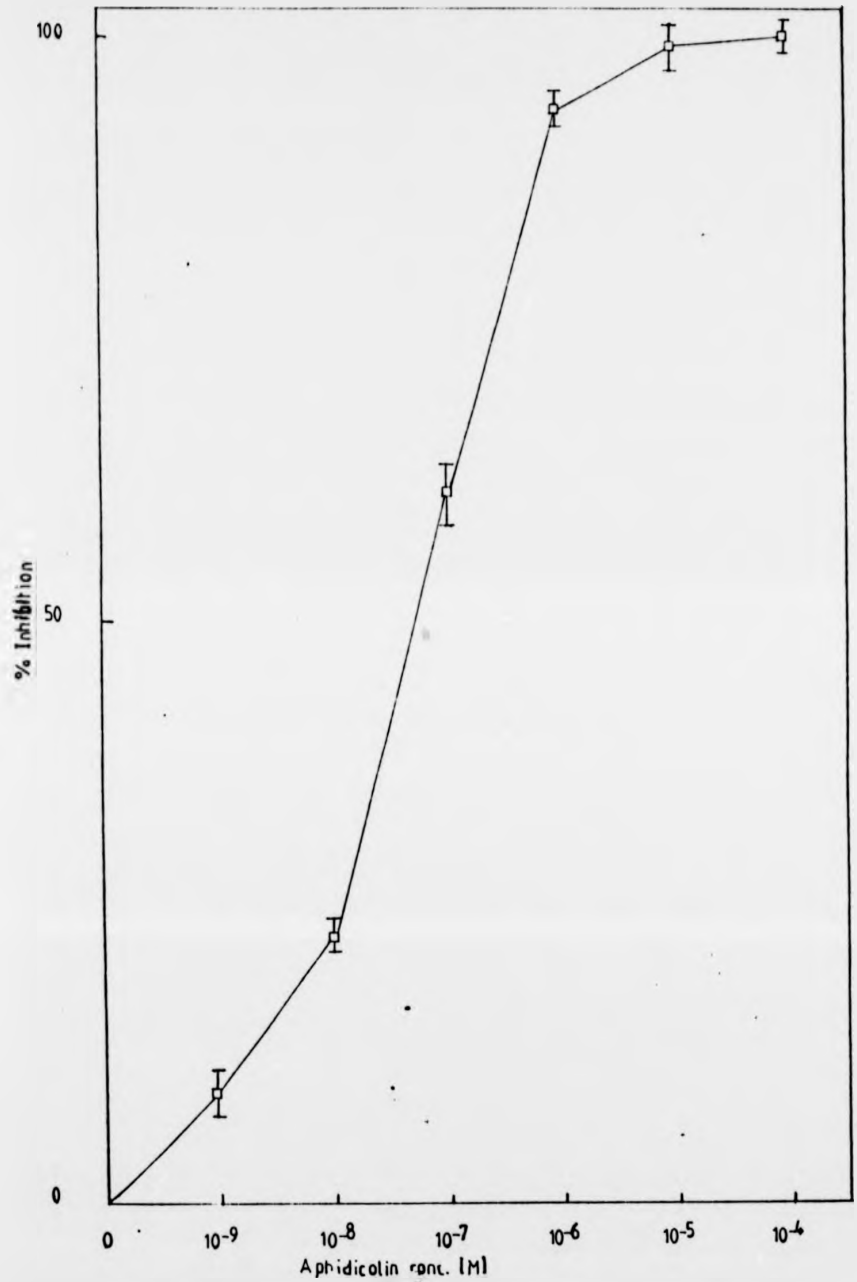



Fig 8.4 The inhibition of $^{3}H$ thymidine uptake in PHA stimulated human peripheral lymphocytes by aphidicolin. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E. n=4

Thymidine incorporation in human lymphocytes was more sensitive to inhibition by cytosine arabinoside, $10^{-8}$ M giving 57% inhibition and $10^{-4}$ M, 97% inhibition. This degree of inhibition is comparable with the effect of araC on thymidine incorporation in Namalwa lymphoblasts (see 3.5.1). The effect of aphidicolin was also investigated at final concentrations of 0, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M. Aphidicolin was added at the same time as the $^{3}$H-thymidine, i.e. 68 hours after PHA stimulation. The lymphocytes were less sensitive to aphidicolin at low concentrations, $10^{-8}$ M giving only 23% inhibition whereas 93% inhibition was obtained with $10^{-6}$ M drug. This result is again comparable with the effect of the drug on Namalwa cells.

### 8.3.2 Thymidine incorporation by PHA stimulated rat lymphocytes

The otpimum PHA concentration required for the stimulation of rat peripheral lymphocytes was determined by measuring thymidine incorporation 72 hours after addition of PHA. The optimum PHA concentration was found to be 25 µg/ml, though stimulation was obtained with concentrations as low as 2.5 µg/ml and as high as 50 µg/ml. Thymidine incorporation was then followed for 96 hours. In order to do this the lymphocytes were pulsed with $^{3}$H-thymidine for one hour at 23, 47, 71 and 95 hours after the addition of PHA. A control experiment was carried out with lymphocytes that had not been exposed to PHA. The results are shown in Fig. 8.5. The period of maximum thymidine incorporation occurs at 72 hours after PHA stimulation after which there is a decrease in the ability of the lymphocytes to incorporate thymidine. As with human lymphocytes there is little thymidine uptake in unstimulated lymphocytes.

Rat peripheral lymphocytes treated with PHA were pulsed with $^{3}$H-thymidine for 4 hours starting 68 hours after PHA stimulation.


Fig 8.5 The thymidine uptake ability of rat peripheral lymphocytes with time after the addition of PHA (25ug/ml) at zero time (□), (○) is a control without PHA. Results are shown as the percentage of the maximum thymidine incorporation into acid precipitable material as described in 3.4.4.

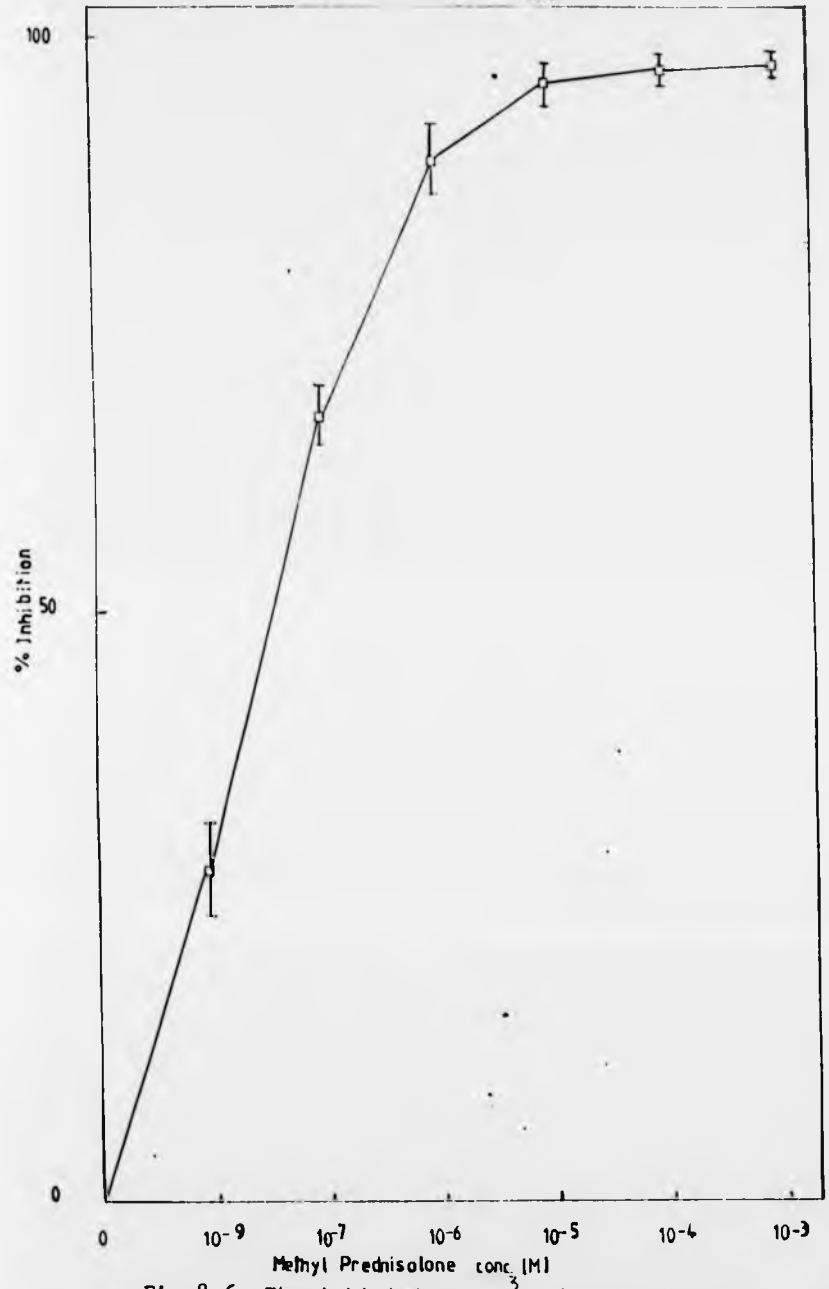



Fig 8.6 The inhibition of $^{3}H$ thymidine uptake in PHA stimulated rat peripheral lymphocytes by 6-α-methyl prednisolone. Results are shown as the percentage inhibition of maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Error bars represent S.E. n=4.

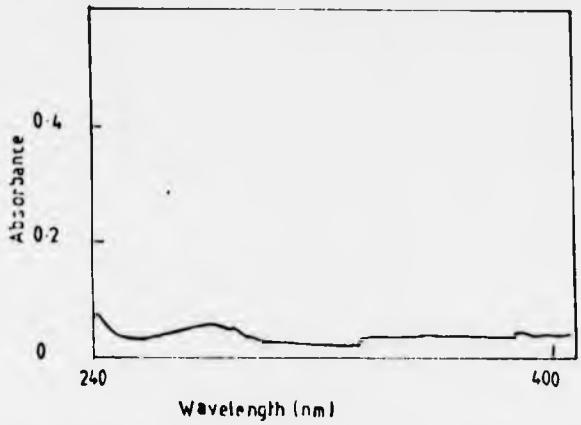
Methyl prednisolone was added to the cultures at the same time as the PHA at final concentrations of 0, $10^{-9}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M. Rat lymphocytes appeared to be more sensitive to the steroid than human lymphocytes as $10^{-9}$ M methyl prednisolone gave 30% inhibition while 90% inhibition was obtained with $10^{-6}$ M methyl prednisolone.

Attempts were made to look at the effects of the topical steroids, clobetasol propionate and clobetasone butyrate, on PHA induced thymidine incorporation by rat peripheral lymphocytes. However both these steroids are water insoluble and no suitable solvent was found at the time that did not itself cause inhibition of the PHA response. In the light of subsequent work on aphidicolin a possible solvent that could have been used is DMSO. This does not have an effect in low concentrations on the PHA response.

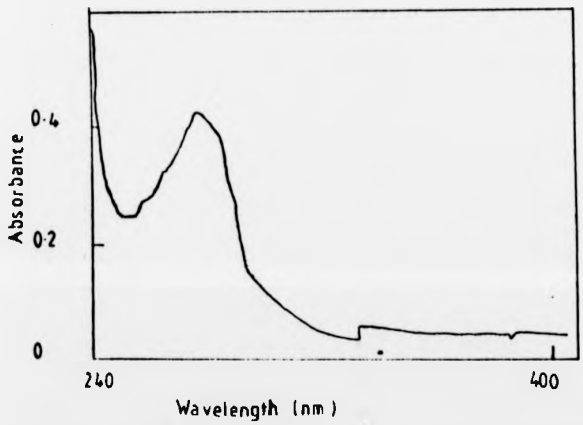
Spleen lymphocytes were easier to isolate than the peripheral lymphocytes isolated from blood and they could be obtained in larger numbers. They showed comparable viability with peripheral lymphocytes (greater than 90% in both cases) when examined using the trypan blue exclusion test. However they showed an apparent resistance to PHA stimulation and so could not be used as a model system in this study.

### 8.3.3 The effect of the oxazolone-rat serum albumin conjugate on rat peripheral lymphocytes

The UV spectra of rat serum albumin, oxazolone and the oxazolone-rat serum albumin conjugate are shown in Figs. 8.7, 8.8 and 8.9 respectively. The original protein concentration of the conjugate was 0.8 mg/ml. This was diluted ten fold to 0.082 mg/ml for the UV spectrum. The oxazolone concentration used was 0.05 mg/ml, i.e. that concentration assuming 100% binding of the oxazolone to the rat serum




8.7a UV spectrum of rat serum albumin 0.082 mg/ml




8.7b UV spectrum of rat serum albumin 0.82 mg/ml

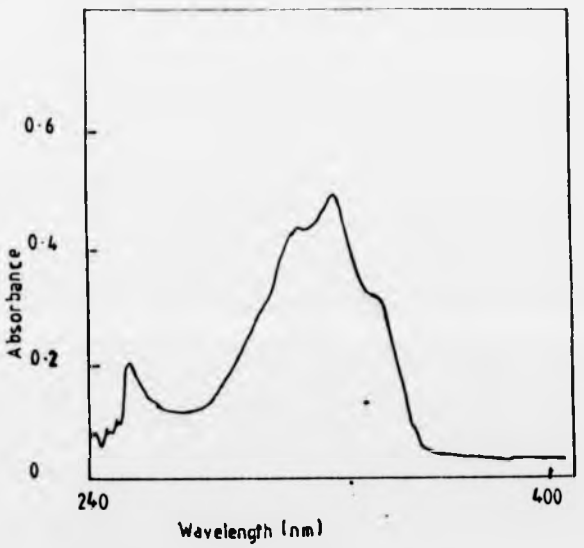



Fig 8.8 UV spectrum of oxazolone 0.05 mg/ml.

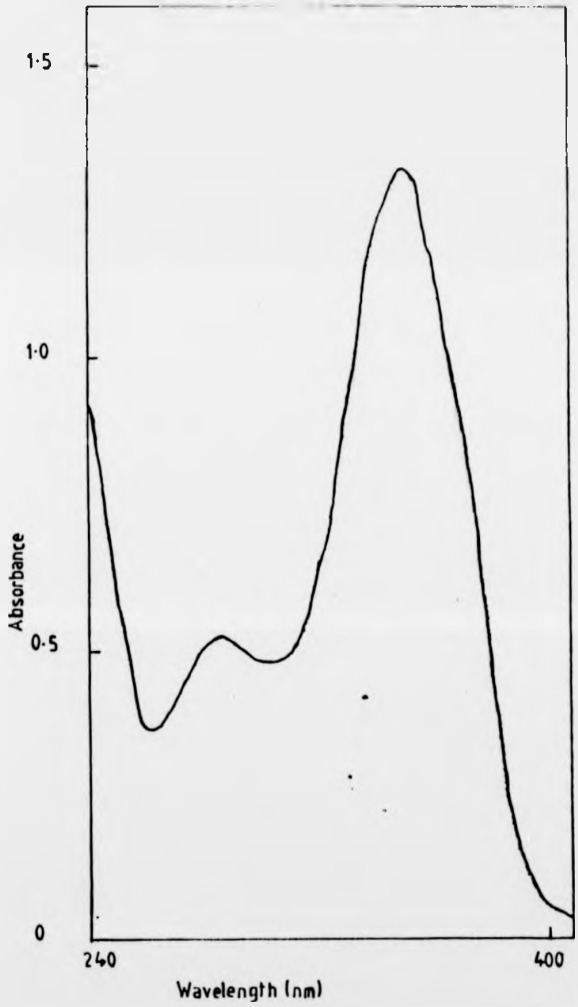



Fig 8.9 UV spectrum of oxazolone - rat serum albumin conjugate 0.082 mg/ml.

albumin. Oxazolone had a maximum absorption at 330 nm. Two spectra were taken for rat serum albumin, one at 0.82 mg/ml and one at 0.082 mg/ml protein concentration as the peak height for the latter at 280 nm was too small to give an accurate measurement. The absorption for the 0.82 mg/ml spectrum was 10 times greater than that for 0.082 mg/ml thus confirming that the relationship between absorbance and concentration was a linear one. The amount of oxazolone bound to rat serum albumin in the conjugate was calculated by using the ratio of the peak heights of oxazolone to rat serum albumin of known concentrations and the ratio of the peak heights at 280 nm and 350 nm in the conjugate (the oxazolone peak was shifted by 20 nm in the conjugate). The peak height at 280 nm in the conjugate was a function of the rat serum albumin concentration, the actual concentration of which was known from the Lowry assay. The oxazolone concentration in the conjugate, which was a function of the height of the 350 nm peak, could therefore be calculated. Using this method the amount of oxazolone bound to the rat serum albumin was calculated to be 130 µg oxazolone/mg protein.

Fig. 8.10 shows the effect of increasing concentrations of the conjugate on thymidine incorporation by rat peripheral lymphocytes. The conjugate was added in place of PHA and thymidine incorporation assayed 72 hours after exposure to the conjugate. This was done by incubating the cultures with $^{3}$H-thymidine for 4 hours, i.e. from 68-72 hours after the addition of the conjugate. Maximum incorporation was achieved using a conjugate concentration of 8.2 µg/ml culture.

In order to show that it was the conjugate that was stimulating the lymphocytes and not oxazolone or rat serum albumin 1 ml cultures of the lymphocytes were incubated with (1) conjugate, 10 µg/ml, this concentration was decided upon after conducting the previous experiment,

| ul conjugate/ ml culture | ug conjugate/ ml culture | % Max activity |
|---|---|---|
| 0 | 0 | 12.3±2.3 |
| 20 | 4.1 | 67 ±1.4 |
| 40 | 8.2 | 100 ±2.3 |
| 80 | 16.4 | 92.3±4.4 |

Fig 8.10 The effect of increasing concentrations of the oxazolone-rat serum albumin conjugate on the thymidine uptake ability of rat peripheral lymphocytes 72 hours after addition of the conjugate to the lymphocyte cultures. Results are shown as the percentage of the maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Errors represent the spread of the results about the mean, n=2

| Additions to culture | % Max. activity |
|---|---|
| 10ug/ml conjugate | 100 ±2.1 |
| 10ug/ml RSA | 5.6±0.3 |
| 5ug/ml oxazolone | 4.9±0.6 |
| Saline | 5.1±0.3 |

Fig 8.11 The measurement of $^{3}$H-thymidine uptake in rat peripheral lymphocytes 72 hours after the addition of i) oxazolone-rat serum albumin conjugate, 10ug/ml, (ii) rat serum albumin, 10ug/ml, (iii) oxazolone, 5ug/ml, and (iv) saline. Results are shown as the percentage of the maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Errors represent the spread of the results about the mean, n=2.

| | |
|---|---|
| 10ug/ml conjugate | 100 ±2.9% |
| 1ug/ml conjugate+ $10^{-4}$M methyl prednisolone | 16 ±2.1% |
| Saline (unstimulated control) | 6.2±1.9% |

Fig 8.12 The inhibition of $^{3}$H-thymidine uptake in oxazolone-protein conjugate stimulated rat peripheral lymphocytes by methyl prednisolone. Results are shown as the percentage of the maximum thymidine incorporation into acid precipitable material as described in 3.4.4. Errors represent the spread of the results about the mean, n=2.

(2) rat serum albumin, 10 µg/ml, (3) oxazolone, 5 µg/ml and (4) a control with an equivalent volume of physiological saline (10 µl). Thymidine incorporation was assayed after 72 hours as before. As can be seen from Fig. 8.11 only the conjugate caused substantial thymidine incorporation.

The effect of 6-α-methyl prednisolone on the stimulation of thymidine incorporation by the oxazolone protein conjugate was investigated. Rat peripheral lymphocyte cultures were incubated with (1) 10 µg/ml of conjugate, (2) 10 µg/ml of conjugate and $10^{-4}$ M methyl prednisolone and (3) a control with an equivalent volume of physiological saline (10 µl). Thymidine incorporation was assayed at 72 hours as previously described. There was no substantial incorporation by the control cultures. Thymidine incorporation by the conjugate-stimulated rat peripheral lymphocytes was inhibited by the steroid, 84% inhibition was obtained with $10^{-4}$ M 6-α-methyl prednisolone.

## 8.4 DISCUSSION

The stimulation of isolated human lymphocytes by PHA to undergo blastogenesis has been well characterised in terms of many of the biochemical responses that occur (see 8.1). It has been shown that a maximum number of lymphocytes undergoing mitosis usually occurs about 72 hours after PHA stimulation. The results obtained here show that thymidine incorporation, used as a measure of DNA synthesis, of PHA stimulated human peripheral lymphocytes occurs 72 hours after stimulation and then rapidly decreases. The maximum DNA synthesis activity at 72 hours after stimulation therefore agrees with similar studies on this system by Loeb *et al.* (181, 182).

The results presented here show that DNA synthesis in PHA

stimulated human peripheral lymphocytes, as measured by thymidine incoporation into acid precipitable material, can be inhibited by 6-α-methyl prednisolone. The results also demonstrate that this system can be used as an *in vitro* model for screening potential immunosuppressive drugs such as the glucocorticoids. Thymidine incorporation is also inhibited by the DNA synthesis inhibitors, cytosine arabinoside and aphidicolin. Whereas steroids have to be administered during the early stages of PHA activation the DNA synthesis inhibitors were administered concurrently with the radioactive thymidine. This difference reflects the different mechanisms of action of the drugs. The steroids appear to act as antagonists to PHA stimulation and in some way affect the molecular processes that initiate blastogenesis. The DNA synthesis inhibitors act during the S phase of the cell cycle by inhibiting DNA polymerase α and therefore their effect is best seen at the period of maximum DNA synthetic activity.

The PHA stimulation of rat peripheral lymphocytes has not been so well characterised in the literature as the PHA stimulation of human lymphocytes therefore this was an important and necessary starting point for the development of the *in vitro* assay system in rat lymphocytes. The optimum PHA concentration was found to be 25 μg/ml and maximum thymidine incorporation occurred at 72 hours after the addition of PHA, i.e. similar to that for human lymphocytes. Thymidine incorporation into DNA by PHA stimulated rat lymphocytes was found to be sensitive to inhibition by methyl prednisolone. This degree of inhibition was comparable with the effect of the drug on PHA stimulated human lymphocytes.

There were several problems inherent in the rat lymphocyte system as compared to the human lymphocyte system. One major problem was that because of the small volume of blood obtainable from one rat

only a small number of cells could be isolated from each blood sample. This made it difficult to carry out accurate dose/response assays over a large drug concentration range. The other major problem was that it was found that rat lymphocytes were often resistant to PHA stimulation unlike human lymphocytes which, generally speaking, are readily stimulated by PHA.

The problem of the resistance of rat spleen lymphocytes to PHA stimulation was not resolved. Though the trypan blue exclusion test is used to determine viability it can only be used as a guide as it only measures one aspect of lymphocyte metabolism. It is possible that the spleen lymphocytes were more sensitive to the preparation procedure than the peripheral lymphocytes and were damaged in some way that was not obviously detectable using the trypan blue test. This damage could have taken the form of damage to the cells' metabolic machinery such as the DNA synthetic machinery as there was no measurable thymidine incorporation following the addition of PHA to the cultures. Alternatively damage to the cell membrane resulting in loss of PHA receptors would also have prevented the PHA response. The presence of a large number of suppressor cells in the culture would also inhibit the response but this is unlikely to be the reason for the lack of response to PHA.

Work with the oxazolone-rat serum albumin conjugate was mainly confined to trying to use the conjugate to stimulate the lymphocytes, rather than as an investigative tool to study the mechanism of blastogenesis and steroid inhibition. A two-pronged approach was adopted in studying lymphocyte stimulation by the conjugate. One approach was to determine an optimal conjugate concentration for lymphocyte stimulation. The other was to ensure that stimulation was actually being caused by the conjugate and not by free oxazolone or rat serum albumin. This was done by setting up cultures containing

oxazolone and albumin as well as the conjugate. It was found that isolated rat peripheral lymphocytes could be stimulated by the conjugate. The stimulation of thymidine incorporation by the conjugate was also shown to be markedly inhibited by 6-α-methyl prednisolone.

# CHAPTER 9

## General Discussion

The work presented in this thesis compares the effects of a number of dissimilar compounds on the DNA metabolism and proliferation of lymphoblasts. The lymphoblast systems that I have used include transformed cell lines derived from both B and T lymphocytes, and mitogen stimulated human and rat peripheral lymphocytes. A protozoan model system was also used. The *in vivo* effect of the inhibitory action of glucocorticoids on lymphocytes was also demonstrated using an animal model system based on the delayed-type hypersensitivity response to oxazolone.

The metabolism of the anti-leukaemia drug, cytosine arabinoside, was investigated in the EBV transformed B lymphoblastoid, Namalwa, cell line, in the CCRF-CEM T lymphoblastoid cell line and in the protozoan *T. pyriformis*. Cytosine arabinoside has to be phosphorylated to its triphosphate by a series of intracellular kinases before it exhibits cytotoxicity (69). However it can also be inactivated by deamination to its inactive derivative uracil arabinoside. The main site of deamination in the *in vivo* situation is the liver, though intracellular deamination may also play a part in inactivating the drug. The mechanism of clinical resistance to araC has not yet been fully defined but it is thought that araC deamination is an important contributory factor (197). One possible way to inhibit the inactivation of the araC by deamination is to administer the deaminase inhibitor tetrahydrouridine in combination with araC.

It has been demonstrated here that araC is a potent inhibitor of DNA synthesis in Namalwa cells, 50% inhibition of thymidine incorporation

into acid precipitable material being obtained with $5 \times 10^{-9}$ M araC. This inhibitory effect is not affected by the addition of tetrahydrouridine. This suggests either (1) there is no araC deaminase present in Namalwa cells or (2) Namalwa cell araC deaminase is resistant to tetrahydrouridine. The metabolites of araC produced by Namalwa cells, CCRF-CEM cells and the protozoan *T. pyriformis* were identified using thin-layer chromatography. The effect of THU on the levels of these metabolites was also investigated. The levels of the enzymes araC deaminase and araC kinase were measured in cell homogenates produced from these cell systems. It was shown that *T. pyriformis* possessed high levels of the deaminase and that this was readily inhibited by THU. There was no deaminase activity present in either the lymphoblastoid cell lines. However the latter possessed high levels of the kinase compared to the protozoan. AraC has a greater inhibitory effect in Namalwa cells than in *T. pyriformis*.

It appears as if the main determining factor for araC to efficiently inhibit DNA synthesis in an *in vitro* system, is a high intracellular kinase level and that high araC deaminase levels may confer resistance to the drug upon an *in vitro* cell system. However care must be taken in extrapolating these results to the *in vivo* clinical situation. The major site of araC deamination is the liver rather than in the leukaemic target cell. It is the plasma level of the drug, coupled with the araC kinase level in target cells that determines its therapeutic efficacy. The plasma levels of araC can be greatly reduced by deamination in the liver. There is little or no intracellular competition between the kinase and deaminase enzymes and intracellular deaminase levels may only play a small part in determining araC efficacy. However inhibition of the liver deaminase may help to increase the araC plasma levels therefore the THU/araC combination may still prove to be effective in maintaining

araC plasma levels and hence increasing araCTP target cell levels.

Despite the limitations involved in using *in vitro* cell systems they can still provide much useful information on the mechanism of araC metabolism and in that respect make useful model systems. One important conclusion derived from this study is that the lack of araC deaminase and the high araC kinase level in Namalwa cells make it an ideal model system for studying the effect of araC on eukaryotic DNA replication.

AraC is a known inhibitor of DNA synthesis and it is generally considered that this is its major mode of action as a cytotoxic agent. The actual cause of cytotoxicity as a result of the inhibition of DNA synthesis is still unclear. Woodcock *et al.*, suggest that araC and other DNA synthesis inhibitors cause cell death by producing severe chromosomal aberrations. These aberrations are postulated to be caused by the reinitiation of previously replicated sements of DNA resulting in the formation of two daughter helices in that section of DNA. This in turn would cause a deformation in the DNA double helix and resultant chromosomal abnormalities (130, 198). Recently Mayor *et al.*, have proposed that cell death is a direct result of the incorporation of araC into internucleotide linkages which in turn causes a decrease in elongation rate (199). From the work presented here it is seen that cytosine arabinoside is incorporated into DNA and that it can also produce a decrease in elongation rate. However the proposition that the decrease in elongation rate is a direct result of araC incorporation and that this is also the cause of cell death seems unlikely in the light of the bulk of the evidence concerning the mechanism of action of araC (see 4.1).

It is generally considered that araC is a competitive inhibitor of DNA polymerase α with respect to dCTP and it is thought to exert

its inhibitory effect on DNA replication primarily in this way (110, 121). Studies on its effect on DNA replication have indicated that it is an inhibitor of DNA chain elongation (125, 126, 127) as would be expected from an inhibitor of DNA polymerase α. In contrast to this Fridland has proposed that at low concentrations of araC its primary effect is as an inhibitor of replicon initiation (128, 129). The results presented in this thesis indicate that even at low concentrations araC is an inhibitor of elongation in Namalwa cells. $5 \times 10^{-9}$ M araC has been shown to slow down the rate of DNA chain elongation. Though an effect on initiation cannot be totally discounted from these results it is suggested that any effect on initiation is a secondary effect caused by the inhibition of elongation. It is possible that the decrease in elongation rate may represent an inhibition of the ligation of DNA pieces between 12 S and 25 S. This cannot be determined from the results presented here but in the light of other evidence concerning the effect of araC on DNA polymerase α it is suggested that any effect on ligation is a secondary effect caused by the inhibition of elongation. The results presented here agree with similar results presented by Dijkwel and Wanka (126) and also agree with the conclusions of Wist *et al.* (125) and Heligren *et al.* (127).

The effect of aphidicolin on DNA replication in Namalwa cells was also investigated. Aphidicolin is an inhibitor of DNA synthesis and 50% inhibition of Namalwa DNA synthesis was obtained with a concentration of $10^{-7}$ M of the compound. It is a known inhibitor of DNA polymerase α (137) and should therefore be a specific inhibitor of DNA chain elongation. The results obtained using sedimentation analysis on alkaline sucrose gradients indicate that this is the case. The gradient profiles obtained are similar to those obtained for araC under similar conditions. This is another indication that araC is

acting at the level of DNA chain elongation.

The work presented by Fridland in favour of araC acting at the level of initiation is not altogether conclusive. He has analysed the DNA produced by CCRF-CEM lymphoblasts in the presence of araC using alkaline sucrose gradient centrifugation. He proposes that an inhibitor of initiation should produce a decrease in the amount of low molecular weight DNA precursors and an accumulation of high molecular weight DNA intermediates and his results appear to show this. However the conditions which he has used to separate the different size-classes of DNA only allow for good separation of high molecular weight DNA intermediates and they do not show the effect of araC clearly on low molecular weight intermediates. When looking at the effect of inhibitors on the process of initiation under these conditions one should look at the formation of low molecular weight primary, Okazaki, pieces of DNA produced immediately after initiation. Fridland however, by only looking at the formation of high molecular weight DNA, is only looking directly at the elongation process. Therefore any conclusions drawn concerning the initiation process must be considered with reservation.

The experimental conditions used that have been described in this thesis allow for the study of the formation of low molecular weight DNA precursors. However in order to study the initiation process directly a more subtle approach must be adopted. A more ideal approach would be to use an isolated nuclei system such as that used by Krokan *et al.* (200), Hershey *et al.* (201, 202) and Brun and Weissbach (45). Krokan *et al.*, have used an isolated HeLa cell nuclei system to investigate the process of initiation and have used this to demonstrate the discontinuous synthesis of DNA via 2-4 S, Okazaki size, pieces of DNA (203). Brun and Weissbach have developed a method for reconstructing a system, capable of synthesising DNA, derived from HeLa cell nuclei (45). They

have used this system to demonstrate the requirement of ribonucleotide triphosphates and the involvement of an RNA polymerase-like enzyme for the initiation of Okazaki fragments. The possibility of deriving an isolated nuclei system from Namalwa cells was investigated during the course of this project.

The proposed method for isolating nuclei was based on that described by Birnie (204). Namalwa cells were lysed in buffer containing 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT and 1% Triton X-100. The lysate was then layered on to 2.2 M sucrose in 10 mM Tris-HCl pH 7-5, 5 mM $MgCl_2$, 0.5 mM DTT, 0.28 M NaCl and centrifuged at 18,000 g, 1 hour, 4°C and the nuclei resuspended in 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5. As yet this system has not been fully developed due to the unavailability of sufficient cells for the preparation of an adequate number of nuclei.

This approach should be used to pursue further the effect of araC on eukaryotic DNA replication. One proposed possible way of doing this would be to make the system limiting with respect to rNTPs so that initiation would cease when all the rNTPs were used up. By adding more rNTPs at this point a DNA replication system that is about to start new rounds of initiation would be achieved. The inhibitory effect of araCTP, the active phosphorylated form of araC, could then be investigated using this system.

In contrast to the specific mechanism of action of the two DNA polymerase inhibitors is the mechanism of action of the phosphoramide mustard derivatives. These bifunctional alkylating agents are chemically very reactive compounds that can interfere with a number of biochemical processes by alkylating biological molecules. They are thought to inhibit cell replication by cross-linking double stranded DNA. The inhibitory effects of phosphoramide mustard,

cyclophosphamide and hydroperoxycyclophosphamide on DNA synthesis in Namalwa cells have been compared. Cyclophosphamide has little effect compared with the other two compounds. $5 \times 10^{-3}$ M cyclophoshamide only gives 20% inhibition of DNA synthesis compared to $5 \times 10^{-3}$ M phosphoramide mustard which gives 65% inhibition and $5 \times 10^{-3}$ M hydroperoxycyclophosphamide which gives 90% inhibition. This differential effect is to be expected as in the *in vivo* situation cyclophosphamide has to be activated by oxidative, hepatic microsomal enzymes. The hydroperoxy compound is an analogue of the activated 4-hydroxycyclophosphamide. Cyclophosphamide, after oxidative activation, breaks down slowly to give phosphoramide mustard which is thought to be the active form of the drug. However the effect of hydroperoxycyclophosphamide is much greater than that of phosphoramide mustard. This may be due to secondary effects of the metabolites of 4-hydroperoxycyclophosphamide.

When the DNA produced by Namalwa cells in the presence of the alkylating agents was analysed on alkaline sucrose gradients DNA intermediates of an unusual size were found. It is suggested that these represent segments of DNA that have been alkylated by the drugs thus producing cross-linked DNA segments of unusual sizes. A more subtle method of investigating this possible cross-linking phenomenon would be to measure density changes in the DNA using CsCl buoyant density centrifugation coupled to incorporation studies with bromodeoxyuridine (BrdUrd). Incorporating BrdUrd into the DNA of synchronised cells results in the production of DNA of a higher density than normal DNA, this can be detected on CsCl gradients. Cross-linking of DNA strands by alkylating agents should enhance this effect by preventing replication and the subsequent incorporation of BrdUrd into DNA.

Another approach would be to use radiolabelled derivatives of

phosphoramide mustard and hydroperoxycyclophosphamide. The binding of the alkylating agents to the DNA molecule could be followed using a similar method to that used for studying the incorporation of araC into DNA. The label should ideally be on the phosphorus atom of the molecule or on one of the carbon atoms on one or both of the alkylating arms. These radioactive derivatives could then be incubated with the target cells and the DNA analysed on alkaline sucrose gradients. The incorporation of the radiolabel into one or more of the DNA intermediates would indicate the interaction of the alkylating agent with DNA.

The study of the immunosuppressive action of glucocorticoids was part of an industrial project concerned with the screening of potential topical anti-inflammatory agents. The objective of the study was two-fold (a) to look at the inhibitory effect of a number of different steroids on an *in vivo* immunological assay, the oxazolone-induced, delayed-type hypersensitivity response and (b) to develop an *in vitro* assay for screening potential immunosuppressive steroids. The results presented show the effect of three different steroids on the DTH response. These were 6-α-methyl prednisolone, clobetasol propionate and clobetasone butyrate. It had already been demonstrated using an *in vivo* assay system for measuring epidermal DNA synthesis in hairless mice that the modification of the C-11 position on the steroid with either an alcohol or a keto group could alter the pharmacological activity of the steroid (152, 154). It was shown using the DTH assay that the alcohol, clobetasol propionate, had a greater inhibitory effect on the DTH response than the ketone, clobetasone butyrate. The topical steroids were also shown to inhibit the DTH response more efficiently that the water soluble steroid, 6-α-methyl prednisolone. The substitution of a halogen at C-9 for a hydrogen has been shown to have an effect on the therapeutic response to imflammation. It has been shown that clobetasone butyrate is more effective than the

unhalogenated steroid, hydrocortisone butyrate, against psoriasis (205). The lack of a halogen at C-9 in 6-α-methyl prednisolone may therefore be the reason for this difference.

The method of administering the drug is also important. Lipophilic steroids that can be applied topically are more desirable as anti-inflammatory agents. A more likely reason for the lower efficiency of 6-α-methyl prednisolone as an inhibitor of the DTH response is that it was administered as an injection into the muscle and therefore had to reach the site of action via the systemic circulation. Therefore it could not be concentrated at the site of the inflammation like the topical steroids.

The DTH assay has several limitations. It is an immunological assay and as such primarily demonstrates the immunosuppressive response of steroids to inflammation. It also does not provide a method for comparing the systemic and local action of topically applied steroids like the hairless mouse assay which was used to show the pronounced systemic effect of clobetasol propionate. It also provides no insight into the cellular mechanism of steroid inhibition.

In order to overcome this latter problem an *in vitro* tissue culture system was set up using PHA stimulated lymphocytes isolated from human blood. PHA selectively acts on T lymphocytes. As the DTH response is primarily a T lymphocyte mediated response the tissue culture system provides a useful *in vitro* analogue of the *in vivo* assay. The lymphocytes were stimulated with PHA and the induction of DNA synthesis was characterised by finding the period of maximum DNA synthetic activity. This was found to occur 72 hours after the addition of PHA to the isolated lymphocyte cultures. DNA synthesis was measured by the incorporation of $^{3}$H-thymidine into acid precipitable material. It was shown that this response to PHA could be inhibited by cytosine

arabinoside, aphidicolon and 6-α-methyl prednisolone. 60% inhibition was obtained with $10^{-6}$ M 6-α-methyl prednisolone, $10^{-7}$ M aphidicolin and $10^{-8}$ M cytosine arabinoside. Cytosine arabinoside and aphidicolin act at the period of maximum DNA synthetic activity since they are S phase specific inhibitors. The steroid however has to be added to the cultures at the same time as the mitogen. The steroids appear to act as antagonists to the PHA response by affecting the molecular processes which are activated by the mitogen. PHA acts by a four step activation process which induces isolated lymphocytes to enter the G1 phase of the cell cycle (176). Steroids act at the G1/S phase of the cell cycle (187) and this is probably one reason why they only inhibit the PHA response when administered soon after mitogen addition. Isolated rat lymphocytes were similarly treated with PHA and the period of maximum DNA synthetic activity after mitogen stimulation was determined. This was found to occur 72 hours after the addition of the mitogen. The response was also shown to be inhibited by 6-α-methyl prednisolone, 90% inhibition being obtained with $10^{-4}$ M of the steroid.

In order to make the *in vitro* assay more representative of the *in vivo* DTH system rat peripheral lymphocytes were treated with an oxazolone-rat serum albumin conjugate instead of PHA. It was shown that this oxazolone-protein conjugate was capable of stimulating DNA synthesis in isolated rat peripheral lymphocytes. This response was inhibited 84% by $10^{-4}$ M 6-α-methyl prednisolone. If developed further this modification of the *in vitro* assay system should provide a useful method for screening potential immunosuppressive glucocorticoids.

Further experiments should be performed in order to fully characterise this system. First of all in order to demonstrate the validity of the *in vitro* assay lymphocytes should be isolated from

rats sensitised to oxazolone and transferred to unsensitised animals. These should then be challenged with oxazolone. If the transferred lymphocytes are the mediators of the DTH response the recipient animals should exhibit a DTH response on being challenged with oxazolone. Another similar transfer experiment that could be performed would be to isolate lymphocytes from unsensitised rats and treat the lymphocytes with the oxazolone-rat serum albumin conjugate. These treated lymphocytes can then be transferred to other unsensitised rats which can then be challenged with oxazolone. If the conjugate is mimicking *in vitro* the contact sensitising effect of oxazolone *in vivo* the animals should exhibit a DTH response on oxazolone challenge.

The system could be further developed by using mouse lymphocytes which can be readily isolated from mouse spleens. A satisfactory method for isolating viable rat and mouse spleen lymphocytes was in the process of being developed but was not carried to a successful conclusion. However, once these problems have been overcome, mouse spleen lymphocytes could provide a useful system as they have been well characterised immunologically in terms of their cell surface antigens. If mouse lymphocytes could be induced to respond to treatment by an oxazolone-mouse serum albumin conjugate then the sub-population of lymphocytes responding to the conjugate could be identified. There are several commercially available radio-immunoassay techniques for identifying the different cell surface antigens on mouse lymphocyte cell membranes. These antigens can be used to identify different mouse lymphocyte populations. If an oxazolone-protein conjugate is mimicking the *in vivo* response to the contact sensitising effect of oxazolone the population of lymphocytes involved should be T lymphocytes.

The work presented in this thesis has demonstrated the diversity of cytotoxic agents that affect the DNA replicative machinery. Some

of these compounds are used clinically as anti-cancer and immuno-suppressive drugs. Their effect on lymphoblasts has been investigated and their different mechanisms of action discussed in the light of the results obtained. A biochemical study of drug action such as this can increase our understanding of the therapeutic activity of drugs and provide a biochemical rationale for their clinical use.

# APPENDIX I

## CALCULATION OF COUNTS DUE TO $^{3}H$ AND $^{14}C$ IN DOUBLE ISOTOPE EXPERIMENTS

When determining the radioactivity of a sample containing two radioactive isotopes each isotope contributes to the total amount of radioactivity. In the case of the $^{3}H$ and $^{14}C$ isotopes each emitts radioactivity of different energies and so their radioactivities can be counted separately on a scintillation counter using two separate channels with different energy limits. However there is always some overlap between the two channels therefore the amount of radioactivity in each channel is the sum of the radioactivity of the major isotope and leakage of radioactivity from the other channel. The counts due to $^{3}H$ and $^{14}C$ in the double labelling experiments described in Chapters 4, 5 and 6 were calculated using the method described below which compensates for this leakage from one channel to another.

It was assumed that there was no leakage from the $^{3}H$ channel to the $^{14}C$ channel so that all the counts in the $^{14}C$ channel are solely the result of the $^{14}C$ isotope. The leakage from the $^{14}C$ channel to the $^{3}H$ channel was calculated using a $^{14}C$ standard. The ratio of counts in the $^{3}H$ channel and $^{14}C$ channel was calculated for the standard, this ratio is called f and had a value of 0.19 under the conditions used.

Let the counts in the $^{14}C$ channel due to the standard be A
Let the counts of the $^{3}H$ channel due to the standard be B
correction factor $f = B/A = 0.19$ for the conditions used.
Total $^{14}C$ counts due to the standard $= A + A.f = A + B$
The counts due to $^{3}H$ and $^{14}C$ for a sample were calculated thus:

Let the counts in the $^{14}C$ channel be A' and the counts in the $^{3}H$ channel be B'.

The total counts due to $^{14}C$ = A' + A'f where A'f is the number of counts due to $^{14}C$ in the $^{3}H$ channel

The total counts due to $^{3}H$ = B' - A'f.

# APPENDIX II

## CALCULATION OF SEDIMENTATION COEFFICIENTS

The size and molecular weight of DNA can be calculated using sedimentation analysis on alkaline sucrose gradients (206). The size of a macromolecule can be defined using a sedimentation coefficient (s) which is defined as

$$s = \frac{1}{\omega^2 r} \cdot \frac{dr}{dt} \qquad (1)$$

where r (cms) is the distance of the molecule from the axis of rotation at time t (seconds) and $\omega$ is the angular velocity of the rotor (radians/second). Sedimentation coefficients are usually expressed in Svedburg units (S), one Svedburg unit is equal to $10^{-13}$ seconds. Sedimentation coefficients are dependent upon the experimental conditions used and the properties of the centrifugation medium therefore all sedimentation coefficients are defined for a set of standard conditions. A standard sedimentation coefficient is obtained for a given macromolecule when sedimenting through water at $20^{\circ}C$ and is expressed as S 20, w. Water is unsuitable as a centrifugation medium so all coefficients are measured in density gradient media and converted to S 20, w. A convenient method for calculating values of S has been devised by McEwen (207). This allows for direct calculations of sedimentation coefficients using a set of precomputed tables. This method relies upon the gradients used being isokinetic. An isokinetic gradient is one in which a constant rotor speed will cause a given macromolecule to move with a constant velocity which is independent of the distance moved. Such a condition will be met if, as the particle sediments, the increased centrifugal force is

balanced by the increased density and viscosity of the gradient medium. These conditions have been shown to exist in a linear, 5%-20% sucrose gradient such as the ones described in this thesis (see Chapter 4) (208).

Under these conditions the S 20, w of a molecule sedimenting at temperature T ($^{o}$C) through a medium of density $\rho T,M$ and viscosity $\eta T,M$

$$S\ 20,\ w\ = \frac{1}{\omega^2 r} \cdot \frac{dr}{dT} \cdot \frac{\eta T,M(\rho P - \rho_{20,w})}{\eta_{20,w}(\rho p - \rho T,M)} \qquad (2)$$

where $\rho_{20,w}$ and $\eta_{20,w}$ are the density and viscosity of water at 20$^{o}$C and $\rho p$ is the density of the sedimenting particle in that medium. For the following calculations the density of DNA was assumed to be 1.2 g/cm$^3$.

Integration of (2) yields

$$S\ 20,\ w \int \omega^2 dt = \int \frac{\eta T,M(\rho P - \rho_{20,w})}{\eta_{20,w}(\rho p - \rho T,M)} \cdot \frac{1}{r} \cdot dr \qquad (3)$$

Assuming that the acceleration and deceleration times are negligible the time integral on the left becomes S 20, w $\omega^2 t$ where t is the total time of centrifugation. When the gradient is linear the radium can be expressed in terms of the sucrose concentration (Z).

$$\frac{dz}{dr} = \frac{Z - Zo}{T} \qquad (4)$$

Where Zo is the extrapolated value of the sucrose concentration at the centre of rotation. Thus the right hand side of the integral may be expressed in terms of the sucrose concentration if the temperature, particle density and Zo are known. If during centrifugation a particle moves from sucrose concentration $Z_1$ and time $t_1$ to a sucrose concnetraton $Z_2$ at time $t_2$ equation (3) can be written as

$$S\ 20,\ w.\omega^2(t_2 - t_1) = I(Z_2) - I(Z_1)$$

balanced by the increased density and viscosity of the gradient medium. These conditions have been shown to exist in a linear, 5%-20% sucrose gradient such as the ones described in this thesis (see Chapter 4) (208).

Under these conditions the S 20, w of a molecule sedimenting at temperature T ($^{o}$C) through a medium of density $\rho T,M$ and viscosity $\eta T,M$

$$S\ 20,\ w\ = \frac{1}{\omega^2 r} \cdot \frac{dr}{dT} \cdot \frac{\eta T,M(\rho P - \rho_{20,w})}{\eta_{20,w}(\rho p - \rho T,M)} \qquad (2)$$

where $\rho_{20,w}$ and $\eta_{20,w}$ are the density and viscosity of water at 20$^{o}$C and $\rho p$ is the density of the sedimenting particle in that medium. For the following calculations the density of DNA was assumed to be 1.2 g/cm$^3$.

Integration of (2) yields

$$S\ 20,\ w \int \omega^2 dt = \int \frac{\eta T,M(\rho P - \rho_{20,w})}{\eta_{20,w}(\rho p - \rho T,M)} \cdot \frac{1}{r} \cdot dr \qquad (3)$$

Assuming that the acceleration and deceleration times are negligible the time integral on the left becomes S 20, w $\omega^2$t where t is the total time of centrifugation. When the gradient is linear the radium can be expressed in terms of the sucrose concentration (Z).

$$\frac{dz}{dr} = \frac{Z - Zo}{T} \qquad (4)$$

Where Zo is the extrapolated value of the sucrose concentration at the centre of rotation. Thus the right hand side of the integral may be expressed in terms of the sucrose concentration if the temperature, particle density and Zo are known. If during centrifugation a particle moves from sucrose concentration $Z_1$ and time $t_1$ to a sucrose concnetraton $Z_2$ at time $t_2$ equation (3) can be written as

$$S\ 20,\ w.\omega^2(t_2 - t_1) = I(Z_2) - I(Z_1)$$

The values of $I(Z_1)$ and $I(Z_2)$ are obtained from McEwens tables (207). Zo can be estimated from the knowledge of the dimensions of the rotor used.

$$Zo = \frac{Z_1 r_2 - Z_2 r_1}{r_2 - r_1}$$

Where $r_1$ is the distance from the axis of rotation of sucrose concentraton $Z_1$ and $r_2$ is the distance from the axis of rotation of sucrose concentration $Z_2$.

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